

PA 321816

PCT / CA 00/01162

JAN 2001 (09 - 01 - 01)

CA 00/01162

REC'D 24 JAN 2001

WIPO

PCT

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

November 07, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/157,807

FILING DATE: October 04, 1999

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)



By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

N. Woodson

N. WOODSON

Certifying Officer

BERESKIN & PARR

BOX 401, 40 KING STREET WEST, TORONTO, CANADA M5H 3Y2
PHONE (416) 364-7311 • FAX (416) 361-1398 • WWW.BERESKINPARR.COMPlease type a plus sign (+) inside this box → ☒PTO/SB/16 (2-98)
Approved for use through 01/31/2001. OMB 0651-0037Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
valid OMB control number.**PROVISIONAL APPLICATION FOR PATENT COVER SHEET**

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)					
Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)			
Curtis Archie John Admir Thor	Braun Purac Borgford	Surrey, British Columbia, Canada Burnaby, British Columbia, Canada New Westminster, British Columbia, Canada			
<input type="checkbox"/> Additional inventors are being named on the ____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (280 characters max) Improved Ricin-Like Toxins for Treatment of Cancer					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
<input checked="" type="checkbox"/> Customer Number		1059		Place Customer Number Bar Code Label here	
OR		Type Customer Number here			
<input type="checkbox"/> Firm or Individual Name		Bereskin & Parr			
Address		Box 401			
Address		40 King Street West			
City		Toronto	State	Ontario	ZIP M5H 3Y2
Country		Canada	Telephone	(416) 364-7311	Fax (416) 361-1398
ENCLOSED APPLICATION PARTS (check all that apply)					
<input checked="" type="checkbox"/> Specification Number of Pages		66		<input type="checkbox"/> Small Entity Statement	
<input checked="" type="checkbox"/> Drawing(s) Number of Sheets		102		<input type="checkbox"/> Other (specify) _____	
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)					
<input checked="" type="checkbox"/> A check or money order is enclosed to cover the filing fees		FILING FEE AMOUNT (\$)			
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:		02-2095		\$150.00	
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input checked="" type="checkbox"/> No.					
<input type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: _____					

Respectfully submitted,

Date October 1/99

SIGNATURE

Micheline Gravelle

TYPED or PRINTED NAME

416-957-1682

TELEPHONE

REGISTRATION NO.

40,261

(if appropriate)

Docket Number.

10447-3

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C., 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C., 20231.

BERESKIN & PARR

UNITED STATES

Title: Improved Ricin-Like Toxins for Treatment of Cancer

Inventors: Curtis Braun, Admir Purac and Thor Borgford

Title: **IMPROVED RICIN-LIKE TOXINS FOR TREATMENT OF
CANCER**

FIELD OF THE INVENTION

5 The invention relates to proteins useful as therapeutics against cancer. The proteins contain A and B chains of a ricin-like toxin linked by a novel linker sequence that is specifically cleaved and activated by proteases specific to cancer.

BACKGROUND OF THE INVENTION

10 Bacteria and plants are known to produce cytotoxic proteins which may consist of one, two or several polypeptides or subunits. Those proteins having a single subunit may be loosely classified as Type I proteins. Many of the cytotoxins which have evolved two subunit structures are referred to as type II proteins
15 (Saelinger, C.B. in Trafficking of Bacterial Toxins (eds. Saelinger, C.B.) 1-13 (CRC Press Inc., Boca Raton, Florida, 1990). One subunit, the A chain, possesses the toxic activity whereas the second subunit, the B chain, binds cell surfaces and mediates entry of the toxin into a target cell. A subset of these toxins kill target cells by inhibiting protein
20 biosynthesis. For example, bacterial toxins such as diphtheria toxin or Pseudomonas exotoxin inhibit protein synthesis by inactivating elongation factor 2. Plant toxins such as ricin, abrin, and bacterial toxin Shiga toxin, inhibit protein synthesis by directly inactivating the ribosomes (Olsnes, S. & Phil, A. in Molecular action of toxins and
25 viruses (eds. Cohen, P. & vanHeyningen, S.) 51-105 Elsevier Biomedical Press, Amsterdam, 1982).

 Ricin, derived from the seeds of *Ricinus communis* (castor oil plant), may be the most potent of the plant toxins. It is estimated that a single ricin A chain is able to inactivate ribosomes at a
30 rate of 1500 ribosomes /minute. Consequently, a single molecule of ricin is enough to kill a cell (Olsnes, S. & Phil, A. in Molecular action of

toxins and viruses (eds. Cohen, P. & vanHeyningen, S.) (Elsevier Biomedical Press, Amsterdam, 1982). The ricin toxin is a glycosylated heterodimer consisting of A and B chains with molecular masses of 30,625 Da and 31,431 Da linked by a disulphide bond. The A chain of
5 ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y. & Tsurugi, K. J., *Biol. Chem.* 262:8128 (1987)). The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and
10 stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al., *Biol. Chem.* 261:7912 (1986)). Once the toxin molecule consisting of the A and B chains is internalized into the cell via clathrin-dependent or independent mechanisms, the greater reduction potential within the cell induces a release of the active A chain, eliciting
15 its inhibitory effect on protein synthesis and its cytotoxicity (Emmanuel, F. et al., *Anal. Biochem.* 173: 134-141 (1988); Blum, J.S. et al., *J. Biol. Chem.* 266: 22091-22095 (1991); Fiani, M.L. et al., *Arch. Biochem. Biophys.* 307: 225-230 (1993)). Empirical evidence suggests that activated toxin (e.g. ricin, shiga toxin and others) in the endosomes is
20 transcytosed through the trans-Golgi network to the endoplasmic reticulum by retrograde transport before the A chain is translocated into the cytoplasm to elicit its action (Sandvig, K. & van Deurs, B., *FEBS Lett.* 346: 99-102 (1994)).

Protein toxins are initially produced in an inactive,
25 precursor form. Ricin is initially produced as a single polypeptide (preproricin) with an amino acid N-terminal presequence and 12 amino acid linker between the A and B chains. The pre-sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M., *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M.,
30 *Eur. J. Biochem.* 146:411-416 (1985)). The proricin is then translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the A and B chains (Lord,

J.M. et al., *FASAB journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is stored in protein bodies inside the plant cells. The A chain is

5 inactive in proricin (O'Hare, M. et al., *FEBS Lett.* 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin (Richardson, P.T. et al., *FEBS Lett.* 255:15-20 (1989)). The ribosomes of the castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as

10 there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell. The exact mechanism of A chain release and activation in target cell cytoplasm is not known (Lord, J.M. et al., *FASAB journal* 8:201-208 (1994)). However, it is known that for activation to take place the disulfide bond between the A and B chains must be reduced and, hence, the linkage between subunits broken.

15 Diphtheria toxin is produced by *Corynebacterium diphtheriae* as a 535 amino acid polypeptide with a molecular weight of approximately 58kD (Greenfield, L. et al., *Proc. Natl. Acad. Sci. USA* 80:6853-6857 (1983); Pastan, I. et al., *Annu. Rev. Biochem.* 61:331-354 (1992); Collier, R.J. & Kandel, J., 1. *Biol. Chem.* 246:1496-1503 (1971)). It is

20 secreted as a single-chain polypeptide consisting of 2 functional domains. Similar to proricin, the N-terminal domain (A-chain) contains the cytotoxic moiety whereas the C-terminal domain (B-chain) is responsible for binding to the cells and facilitates toxin endocytosis. Conversely, the mechanism of cytotoxicity for diphtheria toxin is based

25 on ADP-ribosylation of EF-2 thereby blocking protein synthesis and producing cell death. The 2 functional domains in diphtheria toxin are linked by an arginine-rich peptide sequence as well as a disulphide bond. Once the diphtheria toxin is internalized into the cell, the arginine-rich peptide linker is cleaved by trypsin-like enzymes and the

30 disulphide bond (Cys 186-201) is reduced. The cytotoxic domain is subsequently translocated into the cytosol substantially as described above for ricin and elicits ribosomal inhibition and cytotoxicity.

Pseudomonas exotoxin is also a 66kD single-chain toxin protein secreted by *Pseudomonas aeruginosa* with a similar mechanism of cytotoxicity to that of diphtheria toxin (Pastan, I. et al., *Annu. Rev. Biochem.* 61:331-354 (1992); Ogata, M. et al., *J. Biol. Chem.* 267:25396-25401 (1992); Vagil, M.L. et al., *Infect. Immunol.* 16:353-361 (1977)). *Pseudomonas* exotoxin consists of 3 conjoint functional domains. The first domain Ia (amino acids 1-252) is responsible for cell binding and toxin endocytosis, a second domain II (amino acids 253-364) is responsible for toxin translocation from the endocytic vesicle to the cytosol, and a third domain III (amino acids 400-613) is responsible for protein synthesis inhibition and cytotoxicity. After *Pseudomonas* exotoxin enters the cell, the liberation of the cytotoxic domain is effected by both proteolytic cleavage of a polypeptide sequence in the second domain (near Arg 279) and the reduction of the disulphide bond (Cys 265-287) in the endocytic vesicles. In essence, the overall pathway to cytotoxicity is analogous to diphtheria toxin with the exception that the toxin translocation domain in *Pseudomonas* exotoxin is structurally distinct.

Other toxins possessing distinct functional domains for cytotoxicity and cell binding/toxin translocation include abrin, modeccin and volkensin (Sandvig, K. et al., *Biochem. Soc. Trans.* 21:707-711 (1993)). Some toxins such as Shiga toxin and cholera toxin also have multiple polypeptide chains responsible for receptor binding and endocytosis.

The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains have been described (Rutenber, E. et al. *Proteins* 10:240-250 (1991); Weston et al., *Mol. Bio.* 244:410-422, 1994; Lamb and Lord, *Eur. J. Biochem.* 14:265 (1985); Halling, K. et al. *Nucleic Acids Res.* 13:8019 (1985)). Similarly, the genes for diphtheria toxin and *Pseudomonas* exotoxin have been cloned and sequenced, and the 3-dimensional structures of the toxin proteins have been elucidated and described (Columblatti, M. et al., *J. Biol. Chem.*

261:3030-3035 (1986); Allured, V.S. et al., Proc. Natl. Acad. Sci. USA 83:1320-1324 (1986); Gray, G.L. et al., Proc. Natl. Acad. Sci. USA 81:2645-2649 (1984); Greenfield, L. et al., Proc. Natl. Acad. Sci. USA 80:6853-6857 (1983); Collier, R.J. et al., J. Biol. Chem. 257:5283-5285 (1982)).

5 The potential of bacterial and plant toxins for inhibiting mammalian retroviruses, particularly acquired immunodeficiency syndrome (AIDS), has been investigated. Bacterial toxins such as *Pseudomonas* exotoxin-A and subunit A of diphtheria toxin; dual chain ribosomal inhibitory plant toxins such as ricin, and single chain
10 ribosomal inhibitory -proteins such as trichosanthin and pokeweed antiviral protein have been used for the elimination of HIV infected cells (Olson et al., *AIDS Res. and Human Retroviruses* 7:1025-1030 (1991)). The high toxicity of these toxins for mammalian cells, combined with a lack of specificity of action poses a major problem to
15 the development of pharmaceuticals incorporating the toxins, such as immunotoxins.

 Due to their extreme toxicity there has been much interest in making ricin-based immunotoxins as therapeutic agents for specifically destroying or inhibiting infected or tumourous cells or
20 tissues (Vitetta et al., *Science* 238:1098-1104(1987)). An immunotoxin is a conjugate of a specific cell binding component, such as a monoclonal antibody or growth factor and the toxin in which the two protein components are covalently linked. Generally, the components are chemically coupled. However, the linkage may also be a peptide or
25 disulfide bond. The antibody directs the toxin to cell types presenting a specific antigen thereby providing a specificity of action not possible with the natural toxin. Immunotoxins have been made both with the entire ricin molecule (i.e. both chains) and with the ricin A chain alone (Spooner et al., *Mol. Immunol.* 31:117-125, (1994)).

30 Immunotoxins made with the ricin dimer (IT-Rs) are more potent toxins than those made with only the A chain (IT-As). The increased toxicity of IT-Rs is thought to be attributed to the dual role of

the B chains in binding to the cell surface and in translocating the A chain to the cytosolic compartment of the target cell (Vitetta et al., *Science* 238:1098-1104 (1987); Vitetta & Thorpe, *Seminars in Cell Biology* 2:47-58 (1991)). However, the presence of the B chain in these

5 conjugates also promotes the entry of the immunotoxin into nontarget cells. Even small amounts of B chain may override the specificity of the cell-binding component as the B chain will bind nonspecifically to galactose associated with N-linked carbohydrates, which is present on most cells. IT-As are more specific and safer to use than IT-Rs.

10 However, in the absence of the B chain the A chain has greatly reduced toxicity. Due to the reduced potency of IT-As as compared to IT-Rs, large doses of IT-As must be administered to patients. The large doses frequently cause immune responses and production of neutralizing antibodies in patients (Vitetta et al., *Science* 238:1098-1104 (1987)). IT-As

15 and IT-Rs both suffer from reduced toxicity as the A chain is not released from the conjugate into the target cell cytoplasm.

A number of immunotoxins have been designed to recognize antigens on the surfaces of tumour cells and cells of the immune system (Pastan et al., *Annals New York Academy of Sciences*

20 758:345-353 (1995)). A major problem with the use of such immunotoxins is that the antibody component is its only targeting mechanism and the target antigen is often found on non-target cells (Vitetta et al., *Immunology Today* 14:252-259 (1993)). Also, the preparation of a suitable specific cell binding component may be

25 problematic. For example, antigens specific for the target cell may not be available and many potential target cells and infective organisms can alter their antigenic make up rapidly to avoid immune recognition. In view of the extreme toxicity of proteins such as ricin, the lack of specificity of the immunotoxins may severely limit their usefulness as

30 therapeutics for the treatment of cancer and infectious diseases.

The insertion of intramolecular protease cleavage sites between the cytotoxic and cell-binding components of a toxin can mimic

- the way that the natural toxin is activated. European patent application no. 466,222 describes the use of maize-derived pro-proteins which can be converted into active form by cleavage with extracellular blood enzymes such as factor, Xa, thrombin or collagenase. Garred, O. et al. (J. Biol. Chem. 270:10817-10821 (1995)) documented the use of a ubiquitous calcium-dependent serine protease, furin, to activate shiga toxin by cleavage of the trypsin-sensitive linkage between the cytotoxic A-chain and the pentamer of cell-binding B-units. Westby et al. (Bioconjugate Chem. 3:375-381 (1992)) documented fusion proteins which have a specific cell binding component and protein with a protease sensitive cleavage site specific for factor Xa within the linker sequence. O'Hare et al. (FEBS Lett. 273:200-204 (1990)) also described a recombinant fusion protein of RTA and staphylococcal protein A joined by a trypsin-sensitive cleavage site. In view of the ubiquitous nature of the extracellular proteases utilized in these approaches, such artificial activation of the toxin precursor or immunotoxin does not confer a mechanism for intracellular toxin activation and the problems of target specificity and adverse immunological reactions to the cell-binding component of the immunotoxin remain.
- 20 In a variation of the approach of insertion of intramolecular protease cleavage sites on proteins which combine a binding chain and a toxic chain, Leppla, S.H. et al. (Bacterial Protein Toxins zbl.bakt.suppl. 24:431-442 (1994)) suggest the replacement of the native cleavage site of the protective antigen (PA) produced by *Bacillus anthracis* with a cleavage site that is recognized by cells that contain a particular protease. PA, recognizes, binds, and thereby assists in the internalization of lethal factor (U) and edema toxin (ET), also produced by *Bacillus anthracis*. However, this approach is wholly dependent on the availability of LF, or ET and PA all being localized to cells wherein the modified PA can be activated by the specific protease. It does not confer a mechanism for intracellular toxin activation and presents a

problem of ensuring sufficient quantities of toxin for internalization in target cells.

The *in vitro* activation of a *Staphylococcus*-derived pore forming toxin, (α -hemolysin by extracellular tumour-associated proteases has been documented (Panchel, R.G. et al., *Nature Biotechnology* 14:852-857 (1996)). Artificial activation of α -hemolysin *in vitro* by said proteases was reported but the actual activity and utility of α -hemolysin in the destruction of target cells were not demonstrated.

Hemolysin does not inhibit protein synthesis but is a heptameric transmembrane pore which acts as a channel to allow leakage of molecules up to 3 kD thereby disrupting the ionic balances of the living cell. The α -hemolysin activation domain is likely located on the outside of the target cell (for activation by extracellular proteases). The triggering mechanism in the disclosed hemolysin precursor does not involve the intracellular proteolytic cleavage of 2 functionally distinct domains. Also, the proteases used for the α -hemolysin activation are ubiquitously secreted extracellular proteases and toxin activation would not be confined to activation in the vicinity of diseased cells. Such widespread activation of the toxin does not confer target specificity and limits the usefulness of said α -hemolysin toxin as therapeutics due to systemic toxicity.

A variety of proteases specifically associated with malignancy have been identified and described. For example, cathepsin is a family of serine, cysteine or aspartic endopeptidases and exopeptidases which has been implicated to play a primary role in cancer metastasis (Schwartz, M.K., *Clin. Chim. Acta* 237:67-78 (1995); Spiess, E. et al., *J. Histochem. Cytochem.* 42:917-929 (1994); Scarborough, P.E. et al., *Protein Sci.* 2:264276 (1993); Sloane, B.F. et al., *Proc. Natl. Acad. Sci. USA* 83:2483- 2487 (1986); Mikkelsen, T. et al., *J. Neurosurg* 83:285-290 (1995)). Matrix metalloproteinases (MMPs or matrixins) are zinc-dependent proteinases consisting of collagenases, matrilysin,

stromelysins, gelatinases and macrophage elastase (Krane, S.M., *Ann, N.Y. Acad. Sci.* 732:1-10 (1994); Woessner, J.F., *Ann, N.Y. Acad. Sci.* 732:11-21 (1994); Carvalho, K. et al., *Biochem. Biophys. Res., Comm.* 191:172-179 (1993); Nakano, A. et al. *J. of Neurosurge*, 83:298-307 (1995);

5 Peng, K-W, et al. *Human Gene Therapy*, 8:729-738 (1997); More, D.H. et al. *Gynaecologic oncology*, 65:78-82 (1997)). These proteases are involved in pathological matrix remodeling. Under normal physiological conditions, regulation of matrixin activity is effected at the level of gene expression. Enzymatic activity is also controlled

10 stringently by tissue inhibitors of metalloproteinases (TIMPs) (Murphy, G. et al., *Ann. N.Y. Acad. Sci.*, 732:31-41 (1994)). The expression of MMP genes is reported to be activated in inflamrnatory disorders (e.g. rheumatoid arthritis) and malignancy.

The present inventor has prepared novel recombinant

15 toxic proteins which are specifically toxic to diseased cells but do not depend for their specificity of action on a specific cell binding component. The recombinant proteins toxins have an A chain of a ricin-like toxin linked to a B chain by a synthetic linker sequence which may be cleaved specifically by a protease localised in cells or tissues

20 affected by a specific disease to liberate the toxic A chain thereby selectively inhibiting or destroying the diseased cells or tissues (WO 98/49311 published November 5, 1998 which is incorporated herein by reference).

SUMMARY OF THE INVENTION

25 The present invention relates to novel linker sequences that can be used to prepare recombinant toxic proteins having an A chain of a ricin-like toxin linked to a B chain by the linker sequence. The novel linker sequences of the invention are illustrated in Figures 1-20.

30 In one aspect the present invention provides a purified and isolated nucleic acid encoding a linker sequence comprising: the nucleic acid sequence of pAP301 as shown in Figure 1A; the nucleic acid

sequence of pAP302 as shown in Figure 2A; the nucleic acid sequence of pAP303 as shown in Figure 3A; the nucleic acid sequence of pAP304 as shown in Figure 4A; the nucleic acid sequence of pAP305 as shown in Figure 5A; the nucleic acid sequence of pAP308 as shown in Figure 6A; 5 the nucleic acid sequence of pAP309 as shown in Figure 7A; the nucleic acid sequence of pAP313 as shown in Figure 8A; the nucleic acid sequence of pAP314 as shown in Figure 9A; the nucleic acid sequence of pAP315 as shown in Figure 10A; the nucleic acid sequence of pAP316 as shown in Figure 11A; the nucleic acid sequence of pAP317 as shown in 10 Figure 12A; the nucleic acid sequence of pAP318 as shown in Figure 13A; the nucleic acid sequence of pAP319 as shown in Figure 14A; the nucleic acid sequence of pAP320 as shown in Figure 15A; the nucleic acid sequence of pAP321 as shown in Figure 16A; the nucleic acid sequence of pAP322 as shown in Figure 17A; the nucleic acid sequence of pAP323 as shown in Figure 18A; the nucleic acid sequence of pAP324 as shown in Figure 19A; and the nucleic acid sequence of pAP325 as shown in Figure 20A.

In another aspect, the present invention provides a purified and isolated nucleic acid encoding a recombinant toxic protein 20 comprising (a) a nucleotide sequence encoding an A chain of a ricin-like toxin, (b) a nucleotide sequence encoding a B chain of a ricin-like toxin and (c) a heterologous linker amino acid sequence, linking the A and B chains. The linker sequence is not a native linker sequence of a ricin-like toxin, but rather a synthetic heterologous linker sequence 25 containing a cleavage recognition site for a disease-specific protease. The A and or the B chain may be those of ricin.

The recombinant toxic proteins employing the novel linker sequences of the present invention may be used to treat various forms of cancer including, but not limited to, T- and B-cell 30 lymphoproliferative diseases, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate, cancer and non small cell lung cancer. In an

embodiment, of the invention the cleavage recognition site of the linker is the cleavage recognition site for a cancer-associated protease.

In particular embodiments, the amino acid sequence of the linker comprises the sequence of PAP301 shown in Figure 1C; the
5 sequence of PAP302 shown in Figure 2C; the sequence of PAP303 shown in Figure 3C; the sequence of PAP304 shown in Figure 4C; the sequence of PAP305 shown in Figure 5C; the sequence of PAP308 shown in Figure 6C; the sequence of PAP309 shown in Figure 7C; the sequence of PAP316 shown in Figure 11C; the sequence of PAP317 shown in Figure 12C; the
10 sequence of PAP318 shown in Figure 13C; the sequence of PAP319 shown in Figure 14C; the sequence of PAP323 shown in Figure 18C; the sequence of PAP324 shown in Figure 19C; and the sequence of PAP325 shown in Figure 20C; all cleaved by MMP-9; the sequence of PAP313 shown in Figure 8C; the sequence of PAP314 shown in Figure 9C; the
15 sequence of PAP315 shown in Figure 10C; the sequence of PAP320 shown in Figure 15C; the sequence of PAP321 shown in Figure 16C; the sequence of PAP322 shown in Figure 17C; all cleaved by urokinase-type plasminogen activator.

In a preferred embodiment, the nucleic acid sequences of
20 the recombinant toxic proteins containing ricin A and B chains with each of the linker sequences are shown in Figures 1B, 2B, 3B, 4B, 5B, 6B, 7B, 8B, 9B, 10B, 11B, 12B, 13B, 14B, 15B, 16B, 17B 18B, 19B and 20B.

The present invention also provides a plasmid incorporating the nucleic acid of the invention. In another
25 embodiment, the present invention provides a baculovirus transfer vector incorporating the nucleic acid of the invention.

In a further aspect, the present invention provides a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid
30 sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a cancer-specific protease. The A and/or the B chain may be those of ricin. In an embodiment, the

cleavage recognition site is the cleavage recognition site for a cancer protease substantially as described above. In a particular embodiment, the cancer is T-cell or B-cell lymphoproliferative disease, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate cancer, non
5 small cell lung cancer.

In a further aspect, the invention provides a pharmaceutical composition for treating cancer comprising a recombinant protein of the invention and a pharmaceutically acceptable
10 carrier, diluent or excipient.

In yet another aspect, the invention provides a method of inhibiting or destroying cancer cells, which cancer cells are associated with a specific protease, comprising the steps of preparing a recombinant protein of the invention having a heterologous linker
15 sequence which contains a cleavage recognition site for the cancer specific protease and administering the recombinant protein to the cells. In an embodiment, the cancer is T-cell or B-cell lymphoproliferative disease, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer, prostate
20 cancer, non small cell lung cancer.

The present invention also relates to a method of treating a cancer wherein the cells affected by the cancer are associated with a specific protease by administering an effective amount of one or more recombinant proteins of the invention to an animal in need thereof.

25 Still further, a process is provided for preparing a pharmaceutical for treating cancer wherein cells affected by the cancer are associated with a specific protease comprising the steps of preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin
30 and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for the specific protease; introducing the nucleic acid into a host cell;

expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a 13 chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains wherein the linker sequence contains the cleavage
5 recognition site for the specific protease; and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific
10 examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

15 The invention will be better understood with reference to the drawings in which:

Figure 1A shows the nucleotide sequence of the MMP-9 linker region of pAP301;

20 Figure 1B shows the nucleotide sequence of the pAP301 insert containing ricin and the MMP-9 linker;

Figure 1C shows the amino acid sequence of the PAP301 linker and the wild type ricin linker;

Figure 2A shows the nucleotide sequence of the MMP-9
30 linker region of pAP302;

25 Figure 2B shows the nucleotide sequence of the pAP302 insert containing ricin and the MMP-9 linker;

Figure 2C shows the amino acid sequence of the PAP302 linker and the wild type ricin linker;

30 Figure 3A shows the nucleotide sequence of the MMP-9 linker region of pAP303;

Figure 3B shows the nucleotide sequence of the pAP303 insert containing ricin and the MMP-9 linker;

Figure 3C shows the amino acid sequence of the PAP303 linker and the wild type ricin linker;

Figure 4A shows the nucleotide sequence of the MMP-9 linker region of pAP304;

5 Figure 4B shows the nucleotide sequence of the pAP304 insert containing ricin and the MMP-9 linker;

Figure 4C shows the amino acid sequence of the PAP304 linker and the wild type ricin linker;

10 Figure 5A shows the nucleotide sequence of the MMP-9 linker region of pAP305;

Figure 5B shows the nucleotide sequence of the pAP305 insert containing ricin and the MMP-9 linker;

Figure 5C shows the amino acid sequence of the PAP305 linker and the wild type ricin linker;

15 Figure 6A shows the nucleotide sequence of the MMP-9 linker region of pAP308;

Figure 6B shows the nucleotide sequence of the pAP308 insert containing ricin and the MMP-9 linker;

20 Figure 6C shows the amino acid sequence of the pAP308 linker and the wild type ricin linker;

Figure 7A shows the nucleotide sequence of the MMP-9 linker region of pAP309;

Figure 7B shows the nucleotide sequence of the pAP309 insert containing ricin and the MMP-9 linker;

25 Figure 7C shows the amino acid sequence of the PAP309 linker and the wild type ricin linker;

Figure 8A shows the nucleotide sequence of the UPA linker region of pAP313;

30 Figure 8B shows the nucleotide sequence of the pAP313 insert containing ricin and the UPA linker;

Figure 8C shows the amino acid sequence of the PAP313 linker and the wild type ricin linker;

Figure 9A shows the nucleotide sequence of the UPA linker region of pAP314;

Figure 9B shows the nucleotide sequence of the pAP314 insert containing ricin and the UPA linker;

5 Figure 9C shows the amino acid sequence of the PAP314 linker and the wild type ricin linker;

Figure 10A shows the nucleotide sequence of the UPA linker region of pAP315;

10 Figure 10B shows the nucleotide sequence of the pAP315 insert containing ricin and the UPA linker;

Figure 10C shows the amino acid sequence of the PAP315 linker and the wild type ricin linker;

Figure 11A shows the nucleotide sequence of the MMP-9 linker region of pAP316;

15 Figure 11B shows the nucleotide sequence of the pAP316 insert containing ricin and the MMP-9 linker;

Figure 11C shows the amino acid sequence of the PAP316 linker and the wild type ricin linker;

20 Figure 12A shows the nucleotide sequence of the MMP-9 linker region of pAP317;

Figure 12B shows nucleotide sequence of the pAP317 insert containing ricin and the MMP-9 linker;

Figure 12C shows the amino acid sequence of the PAP317 linker and the wild type ricin linker;

25 Figure 13A shows the nucleotide sequence of the MMP-9 linker region of pAP318;

Figure 13B shows the nucleotide sequence of the pAP318 insert containing ricin and the MMP-9 linker;

30 Figure 13C shows the amino acid sequence of the PAP318 linker and the wild type ricin linker;

Figure 14A shows the nucleotide sequence of the MMP-9 linker region of pAP319;

Figure 14B shows the nucleotide sequence of the pAP319 insert containing ricin and the MMP-9 linker;

Figure 14C shows the amino acid sequence of the PAP319 linker and the wild type ricin linker;

5 Figure 15A shows the nucleotide sequence of the UPA linker region of pAP320;

Figure 15B shows the nucleotide sequence of the pAP320 insert containing ricin and the UPA linker;

10 Figure 15C shows the amino acid sequence of the PAP320 linker and the wild type ricin linker;

Figure 16A shows the nucleotide sequence of the UPA linker region of pAP321;

Figure 16B shows the nucleotide sequence of the pAP321 insert containing ricin and the UPA linker;

15 Figure 16C shows the amino acid sequence of the PAP321 linker and the wild type ricin linker;

Figure 17A shows the nucleotide sequence of the UPA linker region of pAP322;

20 Figure 17B shows the nucleotide sequence of the pAP322 insert containing ricin and the UPA linker;

Figure 17C shows the amino acid sequence of the PAP322 linker and the wild type ricin linker;

Figure 18A shows the nucleotide sequence of the MMP-9 linker region of pAP323;

25 Figure 18B shows the nucleotide sequence of the pAP323 insert containing ricin and the MMP-9 linker;

Figure 18C shows the amino acid sequence of the PAP323 linker and the wild type ricin linker;

30 Figure 19A shows the nucleotide sequence of the MMP-9 linker region of pAP324;

Figure 19B shows the nucleotide sequence of the pAP324 insert containing ricin and the MMP-9 linker;

Figure 19C shows the amino acid sequence of the PAP324 linker and the wild type ricin linker;

Figure 20A shows the nucleotide sequence of the MMP-9 linker region of pAP325;

5 Figure 20B shows the nucleotide sequence of the pAP325 insert containing ricin and the MMP-9 linker;

Figure 20C shows the amino acid sequence of the PAP325 linker and the wild type ricin linker;

10 Figure 21 is a graph showing the treatment of human tumour A431 with PAP304; and

Figure 22 is a graph showing the treatment of human tumour A431 with PAP305.

DETAILED DESCRIPTION OF THE INVENTION

1. Nucleic Acid Molecules of the Invention

15 As mentioned above, the present invention relates to isolated and purified nucleic acid molecules encoding linker sequences. The present invention also relates to isolated and purified nucleic acid molecules encoding a recombinant toxic protein comprising (a) a nucleotide sequence encoding an A chain of a ricin-like toxin, (b) a
20 nucleotide sequence encoding a B chain of a ricin-like toxin and (c) a nucleotide sequence encoding a linker amino acid sequence of the invention, linking the A and B chains. The heterologous linker sequence contains a cleavage recognition site for a cancer-specific protease.

25 The term "isolated and purified" as used herein refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. An "isolated and purified" nucleic acid is also substantially free of
30 sequences which naturally flank the nucleic acid (i.e. sequences located at the 5' and 3' ends of the nucleic acid) from which the nucleic acid is

derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded.

The term "linker sequence" as used herein refers to an internal amino acid sequence within the protein encoded by a nucleic acid molecule of the invention which contains residues linking the A and B chain of a ricin-like toxin so as to render the A chain incapable of exerting its toxic effect, for example catalytically inhibiting translation of an eukaryotic ribosome. The linker sequences of the invention are heterologous to the A and B chain of a ricin-like toxin. By heterologous is meant that the linker sequence is not a sequence native to the A or B chain of a ricin-like toxin or precursor thereof. However, preferably, the linker sequence may be of a similar length to the linker sequence of a ricin-like toxin and should not interfere with the role of the B chain in cell binding and transport into the cytoplasm. When the linker sequence is cleaved the A chain becomes active or toxic.

The nucleic acid molecule of the invention encoding a recombinant toxic protein is cloned by subjecting a preproricin cDNA clone to site-directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene are synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., *Eur. J. Biochem.* 145:266-270 (1985)), several oligonucleotide primers are designed to flank the start and stop codons of the preproricin open reading frame.

The preproricin cDNA is amplified using the upstream primer Ricin-99 or Ricin- 109 and the downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). The purified PCR fragment encoding the preproricin cDNA is, then ligated into an Eco RV-digested pBluescript 11 SK plasmid (Stratagene), and is

used to transform competent XL1-Blue cells (Stratagene). The cloned PCR product containing the putative preproricin gene is confirmed by DNA sequencing of the entire cDNA clone.

5 The preproricin cDNA clone is subjected to site directed mutagenesis; in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). The wild-type preproricin linker region is replaced with the heterogenous linker sequences that are cleaved by the various cancer-specific proteases.

10 The linker regions of the variants encode a cleavage recognition sequence for a cancer-specific protease. The mutagenesis and cloning strategies used to generate the cancer-specific protease-sensitive linker variants are summarized in WO 98149311 to the present inventor. Briefly, the first step involves a DNA amplification using a set of mutagenic primers in combination with the two flanking
15 primers Ricin-109Eco and Ricin1729C Pst I. Restriction digested PCR fragments are gel purified and then ligated with PVL1393 which has been digested with Eco RI and Pst 1. Ligation reactions are used to transform competent XLI-Blue cells (Stratagene). Recombinant clones are identified by restriction digests of plasmid miniprep, DNA and the
20 mutant linker sequences are confirmed by DNA sequencing.

The nucleotide sequences of the novel linker sequences of the invention are as follows: the nucleic acid sequence of pAP301 is shown in Figure 1A; the nucleic acid sequence of pAP302 is shown in Figure 2A; the nucleic acid sequence of pAP303 is shown in Figure 3A;
25 the nucleic acid sequence of pAP304 is shown in Figure 4A; the nucleic acid sequence of pAP305 is shown in Figure 5A; the nucleic acid sequence of pAP308 is shown in Figure 6A; the nucleic acid sequence of pAP309 is shown in Figure 7A; the nucleic acid sequence of pAP313 is shown in Figure 8A; the nucleic acid sequence of pAP314 is shown in
30 Figure 9A; the nucleic acid sequence of pAP315 is shown in Figure 10A; the nucleic acid sequence of pAP316 is shown in Figure 11A; the nucleic acid sequence of pAP317 is shown in Figure 12A; the nucleic acid

sequence of pAP318 is shown in Figure 13A; the nucleic acid sequence of pAP319 is shown in Figure 14A; the nucleic acid sequence of pAP320 is shown in Figure 15A; the nucleic acid sequence of pAP321 is shown in Figure 16A; the nucleic acid sequence of pAP322 is shown in Figure 17A; 5 the nucleic acid sequence of pAP323 is shown in Figure 18A; the nucleic acid sequence of pAP324 is shown in Figure 19A; and the nucleic acid sequence of pAP325 is shown in Figure 20A.

The nucleic acid molecule encoding a recombinant protein of the invention has sequences encoding an A chain of a ricin- 10 like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a cancer-specific protease as described above. The nucleotide sequences encoding the recombinant proteins of the invention are shown in Figures 1B-20B. The nucleic acid may be expressed to provide a recombinant protein 15 having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a disease-specific protease.

The nucleic acid molecule may comprise the A and/or B chain of ricin. The ricin gene has been cloned and sequenced, and the 20 X-ray crystal structures of the A and B chains are published (Rutenber, E., et al. *Proteins* 10:240-250 (1991); Weston et al., *Mol. Biol.* 244:410-422 (1994); Lamb and Lord, *Eur. J. Biochem.* 14:265 (1985); Halling, K., et al., *Nucleic Acids Res.* 13:8019 (1985)). It will be appreciated that the invention includes nucleic acid molecules encoding truncations of A 25 and B chains of ricin like proteins and analogs and homologs of A and B chains of ricin-like proteins and truncations thereof (i.e., ricin-like proteins), as described herein. It will further be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a cDNA of the 30 invention are encompassed by the invention.

Another aspect of the invention provides a nucleotide sequence which hybridizes under high stringency conditions to a

- nucleotide sequence encoding the A and/or B chains of a ricin-like protein. Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989),
- 5 6.3.1 6.3.6. For example, $6.0 \times$ sodium chloride/sodium citrate (SSC) at about 45°C , followed by a wash of $2.0 \times$ SSC at 50°C may be employed.

- The stringency may be selected based on the conditions used in the wash step. By way of example, the salt concentration in the wash step can be selected from a high stringency of about $0.2 \times$ SSC at
- 10 50°C . In addition, the temperature in the wash step can be at high stringency conditions, at about 65°C .

- The nucleic acid molecule may comprise the A and/or B chain of a ricin-like toxin. Methods for cloning ricin-like toxins are known in the art and are described, for example, in E.P. 466,222.
- 15 Sequences encoding ricin or ricin-like A and B chains may be obtained by selective amplification of a coding region, using sets of degenerative primers or probes for selectively amplifying the coding region in a genomic or cDNA library. Appropriate primers may be selected from the nucleic acid sequence of A and B chains of ricin or ricin-like toxins.
- 20 It is also possible to design synthetic oligonucleotide primers from the nucleotide sequences for use in PCR. Suitable primers may be selected from the sequences encoding regions of ricin-like proteins which are highly conserved, as described for example in U.S. Patent No 5,101,025 and E.P. 466,222.

- 25 A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by
- 30 isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., *Biochemistry* 18, 5294-5299 (1979). cDNA is then synthesized from

the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL). It will be appreciated that the methods described above

- 5 may be used to obtain the coding sequence from plants, bacteria or fungi, preferably plants, which produce known ricin-like proteins and also to screen for the presence of genes encoding as yet unknown ricin-like proteins.

- A sequence containing a cleavage recognition site for a
10 specific protease may be selected based on the cancer which is to be targeted by the recombinant protein. The cleavage recognition site may be selected from sequences known to encode a cleavage recognition site for the cancer to be treated. Sequences encoding cleavage recognition sites may be identified by testing the expression product of the sequence
15 for susceptibility to cleavage by the respective protease. A polypeptide containing the suspected cleavage recognition site may be incubated with a protease and the amount of cleavage product determined (Dilannit, 1990, J. Biol. Chem. 285: 17345-17354 (1990)). The protease may be prepared by methods known in the art and used to test suspected
20 cleavage recognition sites.

- The nucleic acid molecule of the invention may be prepared by site directed mutagenesis. For example, the cleavage site of a cancer-specific protease may be prepared by site directed mutagenesis of the homologous linker sequence of a proricin-like toxin. Procedures
25 for cloning proricin-like genes, encoding a linker sequence are described in EP 466,222. Site directed mutagenesis may be accomplished by DNA amplification of mutagenic primers in combination with flanking primers.

- The nucleic acid molecule of the invention may also
30 encode a fusion protein. A sequence encoding a heterologous linker sequence containing a cleavage recognition site for a cancer-specific protease may be cloned from a cDNA or genomic library or chemically

synthesized based on the known sequence of such cleavage sites. The heterologous linker sequence may then be fused in frame with the sequences encoding the A and B chains of the ricin-like toxin for expression as a fusion protein. It will be appreciated that a nucleic acid molecule encoding a fusion protein may contain a sequence encoding an A chain and a B chain from the same ricin-like toxin or the encoded A and B chains may be from different toxins. For example, the A chain may be derived from ricin and the B chain may be derived from abrin. A protein may also be prepared by chemical conjugation of the A and B chains and linker sequence using conventional coupling agents for covalent attachment.

An isolated and purified nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA encoding an A and B chain and a linker into an appropriate vector which allows for transcription of the cDNA to produce an RNA molecule which encodes a protein of the invention. For example, a cDNA can be cloned downstream of a bacteriophage promoter, (e.g. a T7 promoter) in a vector, cDNA can be transcribed in vitro with T7 polymerase, and the resultant RNA can be isolated by standard techniques.

20 II. Novel Linkers and Recombinant Proteins of the Invention

As previously mentioned, the invention provides novel linker sequences. Preferably, the amino acid sequence of the linker is selected from: the amino acid sequence of PAP301 as shown in Figure 1C; the amino acid sequence of PAP302 as shown in Figure 2C; the amino acid sequence of PAP303 as shown in Figure 3C; the amino acid sequence of PAP304 as shown in Figure 4C; the amino acid sequence of PAP305 as shown in Figure 5C; the amino acid sequence of PAP308 as shown in Figure 6C; the amino acid sequence of PAP309 as shown in Figure 7C; the amino acid sequence of PAP313 as shown in Figure 8C; the amino acid sequence of PAP314 as shown in Figure 9C; the amino acid sequence of PAP315 as shown in Figure 10C; the amino acid sequence of PAP316 as shown in Figure 11C; the amino acid sequence of

PAP317 as shown in Figure 12C; the amino acid sequence of PAP318 as shown in Figure 13C; the amino acid sequence of PAP319 as shown in Figure 14C; the amino acid sequence of PAP320 as shown in Figure 15C; the amino acid sequence of PAP321 as shown in Figure 16C; the amino acid sequence of PAP322 as shown in Figure 17C; the amino acid sequence of PAP323 as shown in Figure 18C; the amino acid sequence of PAP324 as shown in Figure 19C; and the amino acid sequence of PAP325 as shown in Figure 20C.

The present invention also provides recombinant proteins which incorporate the A and B chains of a ricin like toxin linked by a heterologous linker sequence containing a cleavage recognition site for a cancer-specific protease as described above. It is an advantage of the recombinant proteins of the invention that they are non-toxic until the A chain is liberated from the B chain by specific cleavage of the linker by the target protease.

The recombinant protein may be used to specifically target cancer cells in the absence of additional specific cell-binding components to target cancer cells. It is a further advantage that the cancer-specific protease cleaves the heterologous linker intracellularly thereby releasing the toxic A chain directly into the cytoplasm of the cancer cell. As a result, said cells are specifically targeted and normal cells are not directly exposed to the activated free A chain.

Ricin is a plant derived ribosome inhibiting protein which blocks protein synthesis in eukaryotic cells. Ricin may be derived from the seeds of *Ricinus communis* (castor oil plant). The ricin toxin is a glycosylated heterodimer with A and B chain molecular masses of 30,625 Da and 31,431 Da respectively. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y; & Tsurugi, K. J. Biol. Chem. 262:8128 (1987)). The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and

stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al., *Biol. Chem.* 261:7912 (1986)).

All protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (preproricin) with a 35 amino acid N-terminal presequence and 12 amino acid linker between the A and B chains. The pre-sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M., *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M., *Eur. J. Biochem.* 146:411-416 (1985)). The proricin is then translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the A and B chains (Lord, J.M. et al., *FASAB journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is stored in protein bodies inside plant cells. The A chain is inactive in the proricin (O'Hare, M., et al., *FEBS Lett.* 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin (Richardson, P.T. et al., *FEBS Lett.* 255:15-20 (1989)). The ribosomes of the castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell.

Ricin-like proteins include, but are not limited to, bacterial, fungal and plant toxins which have A and B chains and inactivate ribosomes and inhibit protein synthesis. The A chain is an active polypeptide subunit which is responsible for the pharmacologic effect of the toxin. In most cases the active component of the A chain is an enzyme. The B chain is responsible for binding the toxin to the cell surface and is thought to facilitate entry of the A chain into the cell cytoplasm. The A and B chains in the mature toxins are linked by disulfide bonds. The toxins most similar in structure to ricin are plant toxins which have one A chain and one B chain. Examples of such

toxins include abrin which may be isolated from the seeds of *Abrus precatorius* and modeccin.

Ricin-like bacterial proteins include diphtheria toxin, which is produced by *Corynebacterium diphtheriae*, *Pseudomonas* enterotoxin A and cholera toxin. It will be appreciated that the term ricin-like toxins is also intended to include the A chain of those toxins which have only an A chain. The recombinant proteins of the invention could include the A chain of these toxins conjugated to, or expressed as, a recombinant protein with the B chain of another toxin.

10 Examples of plant toxins having only an A chain include trichosanthin, MMC and pokeweed antiviral proteins, dianthin 30, dianthin 32, croton II, curcin 11 and wheat germ inhibitor. Examples of fungal toxins having only an A chain include alpha-sarcin, restrictocin, mitogillin, enomycin, phenomycin. Examples of bacterial toxins having only an A

15 chain include cytotoxin from *Shigella dysenteriae* and related Shiga-like toxins. Recombinant trichosanthin and the coding sequence thereof is disclosed in U.S. Patents 5,101,025 and 5,128,460.

In addition to the entire A or B chains of a ricin-like toxin, it will be appreciated that the recombinant protein of the invention may contain only that portion of the A chain which is necessary for exerting its cytotoxic effect. For example, the first 30 amino acids of the ricin A chain may be removed resulting in a truncated A chain which retains toxic activity. The truncated ricin or ricin-like A chain may be prepared by expression of a truncated gene or by

25 proteolytic degradation, for example with Nagarase (Funmatsu et al., *Jap. J. Med. Sci. Biol.* 23:264-267 (1970)). Similarly, the recombinant protein of the invention may contain only that portion of the B chain necessary for galactose recognition, cell binding and transport into the cell cytoplasm. Truncated B chains are described for example in E.P.

30 145,111. The A and B chains may be glycosylated or non-glycosylated. Glycosylated A and B chains may be obtained by expression in the appropriate host cell capable of glycosylation. Non-glycosylated chains

may be obtained by expression in nonglycosylating host cells or by treatment to remove or destroy the carbohydrate moieties.

The proteins of the invention may be prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is compatible with the host cell used. The expression vectors are "suitable for transformation of a host cell", which means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid molecule. Operatively linked is intended to mean that the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory sequences for the transcription and translation of the inserted protein-sequence.

Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal.

Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will
5 also be appreciated that the necessary regulatory sequences may be supplied by the native A and B chains and/or its flanking regions.

The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of
10 the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs, β -galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. Transcription of the
15 selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as β -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant cells can be selected with G418. Cells that have
20 incorporated the selectable marker gene will survive, while the other cells die. This makes it possible to visualize and assay for expression of recombinant expression vectors of the invention and in particular to determine the effect of a mutation on expression and phenotype. It will be appreciated that selectable markers can be introduced on a separate
25 vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target
30 recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target

recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and
5 pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the recombinant protein.

Recombinant expression vectors can be introduced into host cells to produce a transformed host cell. The term "transformed
10 host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a cell by one of many
15 possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran
20 mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

25 Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the proteins of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in
30 Enzymology 185, Academic Press, San Diego, CA (1991).

More particularly, bacterial host cells suitable for carrying out the present invention include *E. coli*, *B. subtilis*, *Salmonella*

typhimurium, and various species within the genus' *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, as well as many other bacterial species well known to one of ordinary skill in the art. Suitable bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the β -lactamase (penicillinase) and lactose promoter system (see Chang et al., *Nature* 275:615 (1978)), the trp promoter (Nichols and Yanofsky, *Meth in Enzymology* 101:155, (1983) and the tac promoter (Russell et al., *Gene* 20:231, (1982)). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Suitable expression vectors include but are not limited to bacteriophages such as lambda derivatives or plasmids such as pBR322 (Bolivar et al., *Gene* 2:95, (1977)), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (see Messing, *Meth in Enzymology* 101:20-77, 1983 and Vieira and Messing, *Gene* 19:259-268 (1982)), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, Calif.).

Typical fusion expression vectors which may be used are discussed above, e.g. pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ). Examples of inducible non-fusion expression vectors include pTrc (Arnann et al., *Gene* 69:301-315 (1988)) and pET 11d (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California, 60-89 (1990)).

Yeast and fungi host cells suitable for carrying out the present invention include, but are not limited to *Saccharomyces cerevisiae*, the genera *Pichia* or *Kluyveromyces* and various species of the genus *Aspergillus*. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari. et al., *Embo J.* 6:229-234 (1987)), pMFa (Kurjan and Herskowitz, *Cell* 30:933-943 (1982)), pJRY88 (Schultz et al., *Gene* 54:113-123 (1987)), and pYES2 (Invitrogen Corporation, San

Diego, CA). Protocols for the transformation of yeast and fungi are well known to those of ordinary skill in the art (see Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929 (1978); Itoh et al., *J. Bacteriology* 153:163 (1983), and Cullen et al. (*BiolTechnology* 5:369 (1987)).

- 5 Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g. ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573) and NS-1 cells. Suitable expression vectors for directing expression in mammalian cells
- 10 generally include a promoter (e.g., derived from viral material such as polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40), as well as other transcriptional and translational control sequences. Examples of mammalian expression vectors include pCDM8 (Seed, B., *Nature* 329:840 (1987)) and pMT2PC (Kaufman et al., *EMBO J.* 6:187-195 (1987)).
- 15 Given the teachings provided herein, promoters, terminators, and methods for introducing expression vectors of an appropriate type into plant, avian, and insect cells may also be readily accomplished. For example, within one embodiment, the proteins of the invention may be expressed from plant cells (see Sinkar et al., *J.*
- 20 *Biosci* (Bangalore) 11:47-58 (1987), which reviews the use of *Agrobacterium rhizogenes* vectors; see also Zambryski et al., *Genetic Engineering, Principles and Methods*, Hollaender and Setlow (eds.), Vol. VI, pp. 253-278, Plenum Press, New York (1984), which describes the use of expression vectors for plant cells, including, among others,
- 25 PAPS2022, PAPS2023, and PAPS2034)

- Insect cells suitable for carrying out the present invention include cells and cell lines from *Bombyx*, *Trichoplusia* or *Spodoptera* species. Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith et al., *Mol.*
- 30 *Cell Biol.* 3:2156-2165 (1983)) and the pVL series (Lucklow, V.A., and Summers, M.D., *Virology* 170:31-39 (1989)). Some baculovirus-insect

cell expression systems suitable for expression of the recombinant proteins of the invention are described in PCT/US/02442.

Alternatively, the proteins of the invention may also be expressed in non-human transgenic animals such as, rats, rabbits, sheep
5 and pigs (Hammer et al. *Nature* 315:680-683 (1985); Palmiter et al. *Science* 222:809-814 (1983); Brinster et al. *Proc. Natl. Acad. Sci. USA* 82:4438-4442 (1985); Palmiter and Brinster *Cell* 41:343-345 (1985) and U.S. Patent No. 4,736,866).

The proteins of the invention may also be prepared by
10 chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, *J. Am. Chem. Assoc.* 85:2149-2154 (1964)) or synthesis in homogenous solution (Houbenweyl, *Methods of Organic Chemistry*, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart (1987)).

15 The present invention also provides proteins comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a cancer-specific protease. Such a protein could be prepared other than by
20 recombinant means, for example by chemical synthesis or by conjugation of A and B chains and a linker sequence isolated and purified from their natural plant, fungal or bacterial source. Such A and B chains could be prepared having the glycosylation pattern of the native ricin-like toxin.

25 N-terminal or C-terminal fusion proteins comprising the protein of the invention conjugated with other molecules, such as proteins may be prepared by fusing, through recombinant techniques. The resultant fusion proteins contain a protein of the invention fused to the selected protein or marker protein as described herein. The
30 recombinant protein of the invention may also be conjugated to other proteins by known techniques. For example, the proteins may be coupled using heterobifunctional thiol-containing linkers as described

in WO 90/10457, N-succinimidyl-3-(2-pyridyldithio-propionate) or N-succinimidyl-5 thioacetate. Examples of proteins which may be used to prepare fusion proteins or conjugates include cell binding proteins such as immunoglobulins, hormones, growth factors, lectins, insulin, low
5 density lipoprotein, glucagon, endorphins, transferrin, bombesin, asialoglycoprotein glutathione-S-transferase (GST), hemagglutinin (HA), and truncated myc.

III. Utility of the Nucleic Acid Molecules and Proteins of the Invention

(a) Therapeutic Methods

10 The recombinant proteins of the invention may be used to specifically inhibit or destroy cancer cells that contain a protease that can cleave the linker sequence of the recombinant protein. It is an advantage of the recombinant proteins of the invention that they have specificity for the cancer cells without the need for a cell binding
15 component. The ricin-like B chain of the recombinant proteins recognize galactose moieties on the cell surface and ensure that the protein is taken up by the cancer cell and released into the cytoplasm. When the protein is internalized into a normal cell cleavage of the heterologous linker would not occur in the absence of the cancer-
20 specific protease and the A chain will remain inactive bound to the B chain. Conversely, when the protein is internalized into a cancer cell, the cancer-specific protease will cleave the cleavage recognition site in the linker thereby releasing the toxic A chain.

Accordingly, the present invention provides a method of
25 inhibiting or destroying cancer cells comprising contacting cancer cells with an effective amount of a recombinant protein or a nucleic acid molecule encoding a recombinant protein of the invention. The present invention also provides a method of treating a cancer comprising administering an effective amount of a recombinant
30 protein or a nucleic acid molecule encoding a recombinant protein of the invention to an animal in need thereof.

The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired result.

5 The term "animal" as used herein means any member of the animal kingdom including all mammals, birds, fish, reptiles and amphibians. Preferably, the animal to be treated is a mammal, more preferably a human.

10 The specificity of a recombinant protein of the invention may be tested by treating the protein with the cancer-specific protease which is thought to be specific for the cleavage recognition site of the linker and assaying for cleavage products. Cancer-specific proteases may be isolated from cancer cells, or they may be prepared recombinantly, for example following the procedures in Darket et al. (*J. Biol. Chem.* 254:2307-2312 (1988)). The cleavage products may be identified for
15 example based on size, antigenicity or activity. The toxicity of the recombinant protein may be investigated by subjecting the cleavage products to an *in vitro* translation assay in cell lysates, for example using Brome Mosaic Virus mRNA as a template. Toxicity of the cleavage products may be determined using a ribosomal inactivation
20 assay (Westby et al., *Bioconjugate Chem.* 3:377-382 (1992)). The effect of the cleavage products on protein synthesis may be measured in standardized assays of *in vitro* translation utilizing partially defined cell free systems composed for example of a reticulocyte lysate preparation as a source of ribosomes and various essential cofactors, such as mRNA
25 template and amino acids. Use of radiolabelled amino acids in the mixture allows quantitation of incorporation of free amino acid precursors into trichloroacetic acid precipitable proteins. Rabbit reticulocyte lysates may be conveniently used (O'Hare, *FEBS Lett.* 273:200-204 (1990)).

30 The ability of the recombinant proteins of the invention to selectively inhibit or destroy cancer cells may be readily tested *in vitro* using cancer cell lines. The selective inhibitory effect of the

recombinant proteins of the invention may be determined, for example, by demonstrating the selective inhibition cellular proliferation in cancer cells or infected cells.

Toxicity may also be measured based on cell viability, for example the viability of cancer and normal cell cultures exposed to the recombinant protein may be compared. Cell viability may be assessed by known techniques, such as trypan blue exclusion assays.

In another example, a number of models may be used to test the cytotoxicity of recombinant proteins having a heterologous linker sequence containing a cleavage recognition site for a cancer associated matrix metalloprotease. Thompson, E.W. et al. (*Breast Cancer Res. Treatment* 31:357-370 (1994)) has described a model for the determination of invasiveness of human breast cancer cells in vitro by measuring tumour cell-mediated proteolysis of extracellular matrix and tumour cell invasion of reconstituted basement membrane (collagen, laminin, fibronectin, Matrigel or gelatin). Other applicable cancer cell models include cultured ovarian adenocarcinoma cells (Young, T.N. et al. *Gynecol. Oncol.* 62:89-99 (1996); Moore, D.H. et al. *Gynecol. Oncol.* 65:78-82 (1997)), human follicular thyroid cancer cells (Demeure, M.J. et al., *World J. Surg.* 16:770-776 (1992)), human melanoma (A-2058) and fibrosarcoma. (HT-1080) cell lines (Mackay, A.R. et al. *Lab. Invest.* 70:781-783 (1994)), and lung squamous (HS-24) and adenocarcinoma (SB-3) cell lines (Spiess, E. et al. *J. Histochem. Cytochem.* 42:917-929 (1994)). An *in vivo* test system involving the implantation of tumours and measurement of tumour growth and metastasis in athymic nude mice has also been described (Thompson, E.W. et al., *Breast Cancer Res. Treatment* 31:357-370 (1994); Shi, Y.E. et al., *Cancer Res.* 53:1409-1415 (1993)).

Although the primary specificity of the proteins of the invention for cancer cells is mediated by the specific cleavage of the cleavage recognition site of the linker, it will be appreciated that specific cell binding components may optionally be conjugated to the proteins

of the invention. Such cell binding components may be expressed as fusion proteins with the proteins of the invention or the cell binding component may be physically or chemically coupled to the protein component. Examples of suitable cell binding components include

5 antibodies to cancer proteins.

Antibodies having specificity for a cell surface protein may be prepared by conventional methods. A mammal, (e.g. a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the peptide which elicits an antibody response in the mammal.

10 Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other
15 immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing
20 cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g. the hybridoma technique originally developed by Kohler and Milstein (*Nature* 256:495-497 (1975))
25 as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., *Immunol.Today* 4:72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., *Monoclonal Antibodies in Cancer Therapy* Allen R., Bliss, Inc., pages 77-96 (1985)), and screening of combinatorial antibody libraries
30 (Huse et al., *Science* 246:1275 (1989)). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated.

The term "antibody" as used herein is intended to include fragments thereof which also specifically react with a cell surface component. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner
5 as described above. For example, F(ab')₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human
10 constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin
15 variable region which recognizes a cell surface antigen (See, for example, Morrison et al., *Proc. Natl Acad. Sci. U.S.A.* 81:6851 (1985); Takeda et al., *Nature* 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., E.P. Patent No. 171,496; European Patent No. 173,494, United Kingdom
20 Patent No. GB 2177096B). It is expected that chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

Monoclonal or chimeric antibodies specifically reactive against cell surface components can be further humanized by producing
25 human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules may be made by techniques known in the art, (e.g. Teng et al., *Proc. Natl.*
30 *Acad. Sci. U.S.A.*, 80:7308-7312 (1983); Kozbor et al., *Immunology Today* 4:7279 (1983); Olsson et al., *Meth. Enzymol.*, 92:3-16 (1982), and PCT Publication W092/06193 or EP 239,400). Humanized antibodies can also

be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments, reactive against cell surface components may also be generated by screening
5 expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with cell surface components. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., *Nature* 341:544-546 (1989); Huse et al., *Science* 246:1275-1281
10 (1989); and McCafferty et al., *Nature* 348:552-554 (1990)). Alternatively, a SCID-hu mouse, for example the model developed by Genpharm, can be used to produce antibodies, or fragments thereof.

(b) Pharmaceutical Compositions

The proteins and nucleic acids of the invention may be
15 formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances
20 may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective., at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active
25 amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be
30 proportionally reduced as indicated by the exigencies of the therapeutic situation.

Accordingly, the present invention provides a pharmaceutical composition for treating cancer comprising a recombinant protein or a nucleic acid encoding a recombinant protein of the invention and a pharmaceutically acceptable carrier, diluent or
5 excipient.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, intramuscular, etc.), oral administration, inhalation, transdermal administration (such as topical cream or ointment, etc.), or suppository
10 applications. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

The compositions described herein can be prepared by per
15 se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's
20 Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the
25 physiological fluids.

The pharmaceutical compositions may be used in methods for treating animals, including mammals, preferably humans, with cancer. It is anticipated that the compositions will be particularly useful for treating patients with B-cell lymphoproliferative disease and
30 melanoma. The dosage and type of recombinant protein to be administered will depend on a variety of factors which may be readily

monitored in human subjects. Such factors include the etiology and severity (grade and stage) of the neoplasia.

The following non-limiting examples are illustrative of the present invention:

5

EXAMPLES

EXAMPLE 1

Cloning and Expression of Proricin Variants Activated by Disease Specific Proteases

Isolation of total RNA

10 The preproricin gene was cloned from new foliage of the castor bean plant. Total messenger RNA was isolated according to established procedures (Sambrook et al., *Molecular Cloning: A Lab Manual* (Cold Spring Harbour Press, Cold Spring Harbour, (1989)) and cDNA generated using reverse transcriptase.

15 cDNA Synthesis

 Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene were synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., *Eur. J. Biochem.*, 145:266-270, 1985), several oligonucleotide primers were
20 designed to flank the start and stop codons of the preproricin open reading frame. The oligonucleotides were synthesized using an Applied Biosystems Model 392 DNA/RNA Synthesizer. First strand cDNA synthesis was primed using the oligonucleotide Ricin1729C. Three micrograms of total RNA was used as a template for oligo
25 Ricin1729C (5'-ATAACTTGCTGCTCCTTTCA-3') primed synthesis of cDNA using Superscript II Reverse Transcriptase (BRL) following the manufacturer's protocol.

DNA Amplification and Cloning

 The first strand cDNA synthesis reaction was used as
30 template for DNA amplification by the polymerase chain reaction (PCR). The preproricin cDNA was amplified using the upstream primer Ricin-99 (5'-CCGGGAGGAAATACTATTGTAAT-3') and the

downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). Amplification was carried out in a Biometra thermal cycler (TRIO-Thermalcycler) using the following cycling parameters: denaturation 95°C for 1 min., annealing 52°C for 1 min., and extension 72°C for 2 min., (33 cycles), followed by a final extension cycle at 72°C for 10 min. The 1846 bp amplified product was fractionated on an agarose gel (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)), and the DNA purified from the gel slice using Qiaex resin (Qiagen) following the manufacturer's protocol. The purified PCR fragment encoding the preproricin cDNA was then ligated (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)) into an Eco RV digested pBluescript II SK plasmid (Stratagene), and used to transform competent XL1-Blue cells (Stratagene). Positive clones were confirmed by restriction digestion of purified plasmid DNA. Plasmid DNA was extracted using a Qiaprep Spin Plasmid Miniprep Kit (Qiagen).

20 DNA Sequencing

The cloned PCR product containing the putative preproricin gene (pAP144) was confirmed by DNA sequencing of the entire cDNA clone. Sequencing was performed using an Applied Biosystems 373A Automated DNA Sequencer, and confirmed by double-stranded dideoxy sequencing by the Sanger method using the Sequenase kit (USB) (see WO 98/49311).

Production and Cloning of Linker Variants

pAP144 cut with EcoRI was used as target for PCR pairs employing the Ricin109-Eco oligonucleotide (Ricin-109Eco primer: 5-GGAGGAATCCGGAGATGAAACCGGGAGGAAATACTATTGTAAT-3) and a mutagenic primer for the 5' half of the linker as well as the Ricin1729PstI primer (Ricin 1729-PstI: 5-

GTAGGCGCTGCAGATAACTTGCTGTCCTTTCAG-3) and a mutagenic primer for the 3' half of the linker. The cycling conditions used for the PCR were 98 degrees C for 2 min.; 98°C 1 min., 52°C 1 min., 72°C 1 min. 15 sec. (30 cycles); 72 degrees C 10 min.; 4 degrees C soak. The PCR products were then digested by EcoRI and PstI respectively, electrophoresed on an agarose gel, and the bands purified by via glass wool spin columns. Triple ligations comprising the PCR product pairs (corresponding halves of the new linker) and pVL1393 vector digested with EcoRI and PstI were carried out. Recombinant clones were identified by restriction digests of plasmid miniprep DNA and the altered linkers confirmed by DNA sequencing. Note that all altered linker variants were cloned directly into the pVL1393 vector.

Isolation of Recombinant Baculoviruses

Insect cells *S. frugiperda* (Sf9), and *Trichoplusia ni* (Tn368 and BTI-TN-581-4 (High Five)) were maintained on EX-CELL 405 medium (JRH Biosciences) supplemented with 10% total calf serum (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Two micrograms of recombinant pVL1393 DNA was co transfected with 0.5 microgram of BaculoGold AcNPV DNA (Pharmingen) into 2×10^6 Tn368 insect cells following the manufacturer's protocol (Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)). On day 5 post-transfection, media were centrifuged and the supernatants tested in limiting dilution assays with Tn368 cells (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Recombinant viruses in the supernatants were then amplified by infecting Tn368 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 3 to 5 supernatants. A total of three rounds of amplification were performed for each recombinant following established procedures (Summers et al., A Manual of Methods of

Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987 and Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)).

5 **Expression of Mutant Proricin**

Recombinant baculoviruses were used to infect 1×10^7 Tn368 or sf9 cells at an moi of 9 in EX-CELL 405 media (JRH Biosciences) with 25mM α -lactose in spinner flasks. Media supernatants containing mutant proricens were collected 3 or 4 days post-infection.

10 **EXAMPLE 2**

Harvesting and affinity column purification of pro-ricin variants

Protein samples were harvested three days post infection. The cells were removed by centrifuging the media at 8288 g for ten minutes using a GS3 (Sorvall) centrifuge rotor. The supernatant was
15 further clarified by centrifuging at 25400 g using a SLA-1500 rotor (Sorvall) for 45 minutes. Protease inhibitor phenylmethylsulfonyl fluoride (Sigma) was slowly added to a final concentration of 1mM. The samples were further prepared by adding α -lactose to a concentration of 20 mM (not including the previous lactose contained in the expression
20 medium). The samples were concentrated to 700 mL using a Prep/Scale-TFF Cartridge (2.5ft, 10K regenerated cellulose (Millipore)) and a Masterflex pump. The samples were then dialysed for 2 days in 1X Column Buffer (50 mM Tris, 100 mM NaCl, 0.02% NaN₃, pH 7.5) using dialysis tubing (10 K MWCO, 32 mm flat width(Spectra/Por)).
25 Subsequently, the samples were clarified by centrifuging at 25400 g using a SLA-1500 rotor (Sorvall) for 45 minutes.

Following centrifugation, the samples were degassed and applied at 4 degrees C to a XK26/20 (Pharmacia) column (attached to a Pharmacia peristaltic pump, Pharmacia Single-path Monitor UV-1
30 Control and Optical Units, and Bromma LKB 2210 2-Channel Recorder) containing 20 mL α -Lactose Agarose Resin (Sigma). The column was

washed for 3 hours with 1X Column buffer. Elution of pro-ricin variant was performed by eluting with buffer (1X Column buffer (0.1% NaN₃), 100 mM Lactose) until the baseline was again restored. The samples were concentrated using an Amicon 8050 concentrator (Amicon) with a
5 YM10 76 mm membrane, utilizing argon gas to pressurize the chamber. The samples were further concentrated in Centricon 10 (Millipore) concentrators according to manufacturer's specifications.

Purification of Variant PAP-Protein by gel filtration chromatography

In order to purify variant from processed material
10 produced during fermentation, the protein was applied to a SUPERDEX 75 (16/60) column and SUPERDEX 200 (16/60) column (Pharmacia) connected in series equilibrated with 100 mM Tris, 200 mM NaCl, pH 7.5 containing 100 mM lactose and 1.0% β -mercaptoethanol (β ME). The flow rate of the column was 0.15 mL/min and fractions were collected
15 every 25 minutes. The UV (280 nm) trace was used to determine the approximate location of the purified PAP-protein and thus determine the samples for Western analysis.

Western analysis of column fractions

Fractions eluted from the SUPERDEX columns
20 (Pharmacia) were analyzed for purity using standard Western blotting techniques. An aliquot of 10 μ L from each fraction was boiled in 1X sample buffer (62.6 mM Tris-Cl, pH 6.8, 4.4% β ME, 2% sodium dodecyl sulfate (SDS), 5% glycerol (all from Sigma) and 0.002% bromophenol blue (Biorad)) for five minutes. Denatured samples were loaded on 12%
25 Tris-Glycine Gels (Biorad) along with 50 ng of RCA₆₀ (Sigma) and 5 μ L of kaleidoscope prestained standards (Biorad). Electrophoresis was carried out for ninety minutes at 100V in 25 mM Tris-Cl, pH 8.3, 0.1% SDS, and 192 mM glycine using the BioRad Mini Protean II cells (Biorad).

30 Following electrophoresis gels were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, and 20%

Methanol) for a few minutes. PVDF Biorad membrane was presoaked for one minute in 100% methanol, rinsed in ddH₂O and two minutes in transfer buffer. Whatman paper was soaked briefly in transfer buffer. Five pieces of Whatman paper, membrane, gel, and another five pieces
5 of Whatman paper were arranged on the bottom cathode (anode) of the Pharmacia Novablot transfer apparatus (Pharmacia). Transfer was for one hour at constant current (2 mA/CM²).

Transfer was confirmed by checking for the appearance of the prestained standards on the membrane. Non-specific sites on the
10 membrane were blocked by incubating the blot for thirty minutes in 1X Phosphate Buffered Saline (1X PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) with 5% skim milk powder (Carnation). Primary antibody rabbit anti-ricin, (Sigma) was diluted 1:3000 in 1X PBS containing 0.1% Tween 20 (Sigma) and 2.5% skim milk
15 and incubated with blot for forty five minutes on a orbital shaker (VWR). Non-specifically bound primary antibody was removed by washing the blot for ten minutes with 1X PBS containing 0.2% Tween 20. This was repeated four times. Secondary antibody donkey anti-rabbit (Amersham) was incubated with the blot under the same
20 conditions as the primary antibody. Excess secondary antibody was washed as described above. Blots were developed with the ECL Western Blotting detection reagents according to the manufacturer's instructions. Blots were exposed to Medtec's Full Speed Blue Film (Medtec) or Amersham's ECL Hyperfilm (Amersham) for one second to
25 five minutes. Film was developed in a KODAK Automatic Developer.

Determination of lectin binding ability of pro-ricin variant

An Immulon 2 plate (VDVR) was coated with 100 µl per well of 10 µg/ml of asialofetuin and left overnight at 4°C. The plate was washed with 3X 300 µL per well with ddH₂O using an automated plate
30 washer (BioRad). The plate was blocked for one hour at 37°C by adding 300 µL per well of PBS containing 1% ovalbumin. The plate was

washed again as above. Pro-ricin variant PAP-protein was added to the plate in various dilutions in 1X Column Buffer, (50 mM Tris, 100 mM NaCl, pH 7.5). A standard curve of RCA₆₀ (Sigma) from 1-10 ng was also included. The plate was incubated for 1 h at 37°C. The plate was washed as above. Anti-ricin monoclonal antibody (Sigma) was diluted 1:3000 in 1X PBS containing 0.5% ovalbumin and 0.1% Tween-20, added at 100 µL per well and incubated for 1 h at 37°C. The plate was washed as above. Donkey-anti rabbit polyclonal antibody was diluted 1:3000 in 1X PBS containing 0.5% ovalbumin, 0.1% Tween-20, and added at 100 µL per well and incubated for 1 h at 37°C. The plate was given a final wash as described above. Substrate was added to plate at 100 µL per well (1 mg/mL o-phenylenediamine (in H₂O), 1 µL/mL H₂O₂) and after development 25 µL of stop solution (20% H₂SO₄) was added and the absorbance read (A490nm-A630nm) using a SPECTRA MAX 340 plate reader (Molecular Devices).

Determination of PAP -Protein activity using the rabbit reticulocyte assay

Ricin samples were prepared for reduction.

- A) RCA₆₀ = 3,500 ng/µL of RCA₆₀ + 997 µL 1x Endo buffer (25 mM Tris, 25 mM KCl, 5mM MgCl₂, pH 7.6)
Reduction = 95 µL of 10 ng/µL + 5 µL β-mercaptoethanol
- B) Ricin variants
Reduction = 40 µL variant + 2 µL β-mercaptoethanol
The ricin standard and the variants were incubated for 30 minutes at room temperature.

Ricin - Rabbit Reticulocyte lysate reaction

The required number of 0.5 mL tubes were labelled. (2 25 tubes for each sample, + and - aniline). To each of the sample tubes 20 µL of 1X endo buffer was added, and 30 µL of buffer was added to the

controls. To the sample tubes either 10 μ L of 10ng/ μ L, Ricin or 10 μ L of variant was added. Finally, 30 μ L of rabbit reticulocyte lysate was added to all the tubes. The samples were incubated for 30 minutes at 30°C using the thermal block. Samples were removed from the 0.5 mL tube and contents added into a 1.5 mL tube containing 1 mL of TRIZOL (Gibco). Samples were incubated for 15 minutes at room temperature. After the incubation, 200 μ L of chloroform was added, and the sample was vortexed and spun at 12,000 g for 15 minutes at 4°C. The top aqueous layer from the samples was removed and contents added to a 1 mL tube containing 500 μ L of isopropanol. Samples were incubated for 15 minutes at room temperature and then centrifuged at 12,000 for 15 minutes at 4°C. Supernatant was removed and the pellets were washed with 1 mL of 70% ethanol. Centrifugation at 12,000 g for 5 minutes at 4°C pelleted the RNA. All but approximately 20 μ L of the supernatant was removed and the RNA pellet was allowed to air dry. Pellets from the other samples (+aniline samples) were dissolved in 20 μ L of DEPC treated ddH₂O. An 80 μ L aliquot of 1 M aniline (distilled) with 2.8 M acetic acid was added to these RNA samples and transferred to a fresh 0.5 mL tube. The samples were incubated in the dark for 3 minutes at 60°C. RNA was precipitated by adding 100 μ L of 95% ethanol and 5 μ L of 3M sodium acetate, pH 5.2 to each tube and centrifuging at 12,000 g for 30 minutes at 4°C. Pellets were washed with 1 mL 70% ethanol and centrifuged again at 12,000g for 5 minutes at 4°C to precipitate RNA. The supernatant was removed and air dried. These pellets were dissolved in 10 μ L of 0.1 X E buffer. To all samples, 10 μ L of formamide loading dye was added. The RNA ladder (BRL) (8 μ L of ladder + 8 μ L of loading dye) was also included. Samples were incubated for 2 minutes at 70°C on the thermal block. Electrophoresis was carried out on the samples using 1.2% agarose, 50% formamide gels in 0.1X E buffer +

0.2% SDS. The gel was run for 90 minutes at 75 volts. RNA was visualized by staining the gel in 1 µg/µL ethidium bromide in running buffer for 45 minutes. The gel was examined on a 302 nm UV box, photographed using the gel documentation system and saved to a computer disk.

Results:

Protein Expression Yields

Aliquots were taken at each stop of the harvesting/purification and tested. Yields of functional ricin variant were determined by ELISA. Typical results of an 2400 mL prep of infected T. *ni* cells are given below.

<u>Aliquot</u>	<u>µg PAP 220</u>
Before concentration and dialysis	6000
after concentration and dialysis	4931
15 alpha- Lactose agarose column flow through	219
alpha- Lactose agarose column elution	1058

Yield: $1058/6000 = 17.6\%$

Purification of PAP-Protein and Western Analysis of column fractions

Partially purified PAP-protein was applied to Superdex 75 and 200 (16/60) columns connected in series in order to remove the contaminating non-specifically processed PAP-protein. Eluted fractions were tested via Western analysis as described above and the fractions containing the most pure protein were pooled, concentrated and dialyzed against 1 X PBS buffer and then sterilized by filtration (Millipore). Final purified PAP-protein has less than 1% processed variant.

The purified PAP-protein was tested for susceptibility to cleavage by the particular protease and for activation of the A-chain of the pro-ricin variant, (inhibition of protein synthesis). Typically, PAP protein was incubated with and without protease for a specified time

period and then electrophoresed and blotted. Cleaved PAP will run as two 30 kDa proteins (B is slightly larger) under reducing (SDS-PAGE) conditions. Unprocessed PAP-protein, which contains the linker region, will migrate at 60 kDa.

5 **Activation of PAP -Protein variant with Specific Protease**

Activation of protease treated PAP-protein is based on the method of *May et al.* (EMBO Journal. 8 301-8, 1989). Activation of ricin A chain upon cleavage of the intermediary linker results in catalytic depurination of the adenosine 4325 residue of 28S or 26S rRNA. This
10 depurination renders the molecule susceptible to amine-catalyzed hydrolysis by aniline of the phosphodiester bond on either side of the modification site. The result is a diagnostic 390 base band. As such, reticulocyte ribosomes incubated with biochemically purified ricin A chain, released the characteristic RNA fragment upon aniline treatment
15 of isolated rRNA (May, M.J. et al. Embo. Journal, 8:301-308 at 302- 303 (1989)). It is on this basis that the assay allows for the determination of activity of a ricin A chain which has been cleaved from the intact unit containing a particular variant linker sequence.

EXAMPLE 3

20 **In Vitro Protease Digestion of Proricin Variants:**

Affinity-purified proricin variant is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. Ricin- like toxin variants are eluted from the lactose-agarose matrix in protease digestion buffer (50mM NaCl, 50mM Na-
25 acetate, pH 5.5, 1mM dithiothreitol) containing 100mM lactose. Proricin substrate is then incubated at 37°C for 60 minutes with a disease-specific protease. The cleavage products consisting ricin A and B chains are identified using SDS/PAGE (Sambrook et al., Molecular Cloning: a Laboratory Manual, 2nd. ed., Cold Spring Harbor Press, 1989), followed
30 by Western blot analysis using anti- ricin antibodies (Sigma).

Matrix metalloproteinases may be prepared substantially as described by Lark, M.W. et al. (*Proceedings of the 4th International*

Conference of the Inflammation Research Association Abstract 145 (1988)) and Welch, A.R. et al. (Arch. Biochem. Biophys. 324:59-64 (1995)).

- Urokinase plasminogen activator may be prepared substantially as described by Holmberg, L. et al. (Biochim Biophys Acta, 445:215-222, (1976)) and Someno, T. et al. (J Biochem 97:1493-1500 (1985)).

EXAMPLE 4

Cytotoxicity of Ricin and Ricin Variants on Cell Lines

Cell Lines

COS-I (African Green Monkey Kidney Cells)

- 10 This is an SV40 transformed cell line which was prepared from established simian cells CV-1. (Reference: Gluzman, Y. (1975) Cell, 23, 175 - 182)(ATCC CRL 1650)

HT-1080 Human Fibrosarcoma

- (ATCC CCL 121) This cell line was shown to produce
15 active MMP-9 in tissue culture. References: Moore et al. (1997) Gynecologic Oncology 65, 83-88.

Cell Preparation

- After washing with 1XPBS (0.137 M NaCl, 2.68 mM KCl, 8.10 mM Na₂HP0₄, 1.47 mM KH₂PO₄), cells in log phase growth were
20 removed from plates with 1X trypsin/EDTA (Gibco/BRL). The cells were centrifuged at 1100 rpm for 3 min, resuspended in Dulbecco's Modified Eagle Medium containing 10%FBS and 1X pen/strep, and then counted using a haemocytometer. They were adjusted to a concentration of 5 X 10⁴ cells•ml⁻¹. One hundred microliters per well of
25 cells was added to wells 2B - 2G through to wells 9B - 9G of a Falcon 96 well tissue culture plate. A separate 96 well tissue culture plate was used for each sample of Ricin or Ricin variant. The plates were incubated at 37°C with 5% CO₂ for 24 hours.

Toxin Preparation

- 30 The Ricin and Ricin variants were sterile filtered using a 0.22 µm filter (Millipore). The concentration of the sterile samples were

then quantified by A_{280} and confirmed by BCA measurements (Pierce). For the variants digested with the MMP-9 protease in vitro, the digests were carried out as described in the digestion procedure for each protease. The digests were then diluted in the $1000 \text{ ng} \cdot \text{ml}^{-1}$ dilution and sterile filtered. Ricin and Ricin variants were serially diluted to the following concentrations: $1000 \text{ ng} \cdot \text{ml}^{-1}$, $100 \text{ ng} \cdot \text{ml}^{-1}$, $10 \text{ ng} \cdot \text{ml}^{-1}$, $1 \text{ ng} \cdot \text{ml}^{-1}$, $0.1 \text{ ng} \cdot \text{ml}^{-1}$, $0.01 \text{ ng} \cdot \text{ml}^{-1}$, $0.001 \text{ ng} \cdot \text{ml}^{-1}$ with media containing 10%FBS and 1X pen/strep.

Application of Toxin or Variants to Plates

Columns 2 to 9 were labeled: control, $1000 \text{ ng} \cdot \text{ml}^{-1}$, $100 \text{ ng} \cdot \text{ml}^{-1}$, $10 \text{ ng} \cdot \text{ml}^{-1}$, $1 \text{ ng} \cdot \text{ml}^{-1}$, $0.1 \text{ ng} \cdot \text{ml}^{-1}$, $0.01 \text{ ng} \cdot \text{ml}^{-1}$, $0.001 \text{ ng} \cdot \text{ml}^{-1}$ consecutively. The media was removed from all the sample wells with a multichannel pipettor. For each plate of variant and toxin, $50 \mu\text{l}$ of media was added to wells 2B to 2G as the control, and $50 \mu\text{l}$ of each sample dilution was added to the corresponding columns. The plates were incubated for one hour at 37°C with 5% CO_2 , then washed once and replaced with media, then incubated for 48 hours at 37°C with 5% CO_2 .

Sample Application

The whole amount of media (and/or toxin) was removed from each well with a multichannel pipettor, and replaced with $100 \mu\text{l}$ of the substrate mixture (Promega Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit). The plates were incubated at 37°C with 5% CO_2 for 2 to 4 hours, and subsequently read with a Spectramax 340 96 well plate reader at 490nm . The IC_{50} values were calculated using the GRAFIT software program.

Results

The results of the cytotoxicity assay are shown in Tables 1 to 4. In almost all cases the novel variants show preferential activation in the tumour cell line HT1080 (human fibrosarcoma) as compared

with the non-tumourogenic cell line COS-1 (immortalized cell line from the kidney of an African green monkey).

EXAMPLE 5

Maximum Tolerable Dose Data - PAP304 and PAP305

5 The protocol for the maximum tolerable dose (MTD) study involved a single intravenous injection of either PAP304 or PAP305 into the tail vein of either a Nude/SCID mouse. Three animals were used for each dose tested. The samples were diluted into saline solution containing 100 µg/mL Bovine Serum Albumin on the same
10 day as the injection. Animals were observed for 14 days after dosing. Any surviving animals were euthanized after 14 days of study. The MTD value was defined as the highest dose of sample tested where all animals in the group survived.

PAP304 - 150 µg/kg

15 PAP305 - 25 µg/kg

(cf. Ricin - 1.6 µg/kg and PAP220 - 13 µg/kg)

These results demonstrate that the pAP304 and pAP305 linkers decrease the toxicity of the recombinant proteins.

EXAMPLE 6

In vivo Studies

(a) Protocol for A431 Animal Model Studies

Tumour growth will be monitored daily by measuring tumour dimensions with calipers. The treatment initiation date is dependent on the rate of tumour growth. When four groups (4 mice
25 per group) of mice develop tumours of the desired size (50 MM³ - 100 MM³) the mice will be weighed and the treatment will be initiated. This treatment initiation date is considered as day 1, and the mice will be given a bolus intravenous injection of PAP304 on this day. Injections will be administered through the lateral tail vein. The
30 treatment groups will be as shown in Table 5.

All samples and buffer will be made up in saline solution containing 100 µg/mL Bovine Serum Albumin.

(b) In Vivo Efficacy Studies

Subcutaneous A431 tumours were established in SCID mice. The tumours were treated with either PAP304 or PAP305 when the tumours reached 50 MM³ on Days 1, 5 and 9. The results shown in Figures 21 and 22 demonstrate that the linker decreases the toxicity of the variant (as compared with ricin) and the variants PAP304 and PAP305 are activated at or near the A431 (human epithelial carcinoma) solid tumour in mice. A very exciting result is shown in Figure 21. In this study, the variant PAP304 was able to slow down the growth of A431 solid tumour (17 day delay), without any signs of dose limiting toxicity (e.g., no weight loss or death).

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims:

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

**FULL CITATIONS FOR CERTAIN REFERENCES REFERRED TO IN
THE SPECIFICATION**

- 5 Bever Jr., C.T., Panitch, H.S., and Johnson, K.P. (1994) *Neurology* 44(4), 745-8. increased cathepsin B activity in peripheral blood mononuclear 5 cells of multiple sclerosis patients.

Cohen, P., Graves, H.C., Peehl, D.M., Kamarei, M., Giudice, L.C., and Rosenfeld, R.G. (1992) *Journal of Clinical Endocrinology and Metabolism* 75(4), 1046-53. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma.

- 10 Conover, C.A. and De Leon, D.D. (1994) *J. Biol. Chem.* 269(10), 7076-80. Acid activated insulin-like growth factor-binding protein-3 proteolysis in normal and transformed cells. Role of cathepsin D.

- 15 Hansen, G., Schuster, A., Zubrod, C., and Wahn, V. (1995) *Resp* 62(3), 117-24. Alpha 1-proteinase inhibitor abrogates proteolytic and secretagogue activity of cystic fibrosis sputum.

- 20 Muller, H.L., Oh, Y., Gargosky, S.E., Lehrnbecher, T., Hintz, R.L., and Rosenfeld, R.G. (1993) *Journal of Clinical Endocrinology and Metabolism* 77(5), 1113-9. Concentrations of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3), IGF, and IGFBP-3 protease activity in cerebrospinal fluid of children with leukemia, central nervous system tumor, or meningitis.

1) Cytotoxicity of Selected Variants

Table 1: Selected Variants against COS-1 Cells – Target Protease MMP-9

	Ricin	PAP220	PAP301	PAP302	PAP303	PAP304	PAP305	PAP308
Linker Length (residues)	-	23	23	16	15	8	12	12
Reduction in toxicity relative to Ricin	1X	23X	24X	118X	63X	1220X	145X	89X

Table 2: Selected Variants against HT1080 Cells – Target Protease MMP-9

	Ricin	PAP220	PAP301	PAP302	PAP303	PAP304	PAP305	PAP308
Linker Length (residues)	-	23	23	16	15	8	12	12
Reduction in toxicity relative to Ricin	1X	4X	5X	24X	12X	137X	38X	21X

2) Cytotoxicity Data from Selected Variants

Table 3: Selected Variants against COS-1 Cells

	Ricin	PAP313 a)	PAP314 a)	PAP315 a)	PAP316 b)	PAP317	PAP318 b)	PAP319
Linker Length (residues)	-	7	15	14	23	21	22	23
Reduction in toxicity relative to Ricin	1X	72X	52X	75X		55X	100X	155X

	Ricin	PAP320	PAP321	PAP322	PAP323	PAP324	PAP325	
Linker Length (residues)	-			9 a)	21 b)	19 b)	17 b)	
Reduction in toxicity relative to Ricin	1X			82X	65X	67X	82X	

a) Target protease Urokinase Plasminogen Activator (UPA)

b) Target protease MMP-9

Table 4: Selected Variants against HT1080 Cells

	Ricin	PAP3 13a)	PAP3 14a)	PAP3 15a)	PAP3 16b)	PAP3 17	PAP3 18b)	PAP3 19
Linker Length (residues)	-	7	15	14	23	21	22	23
Reduction in toxicity relative to Ricin	1X	161X	27X	18X		9X	51X	49X

	Ricin	PAP320	PAP321	PAP322 a)	PAP323 b)	PAP324 b)	PAP325 b)	
Linker Length (residues)	-			9	21	19	17	
Reduction in toxicity relative to Ricin	1X			51X	15X	14X	20X	

a) Target protease Urokinase Plasminogen Activator (UPA)

b) Target protease MMP9

Table 5

Group	Sample	Drug Dose ($\mu\text{g/kg}$)	Treatment (days)
1	Control - Buffer	0	1, 5, and 9
2	PAP304	75	1, 5, and 9
3	PAP304	100	1, 5, and 9
4	PAP304	150	1, 5, and 9

WE CLAIM:

1. A purified and isolated nucleic acid molecule comprising (a) a nucleotide sequence encoding an A chain of a ricin-like toxin, (b) a nucleotide sequence encoding a B chain of a ricin-like toxin and (c) a
5 nucleotide sequence encoding a heterologous linker amino acid sequence linking the A and B chains, the heterologous linker sequence containing a cleavage recognition site for a cancer-specific protease.
2. A nucleic acid molecule of claim 1 wherein the cleavage recognition site is recognized by a cancer-associated protease which is
10 selected from the group consisting of a matrix metalloproteinase and a urokinase-type plasminogen activator.
3. A nucleic acid molecule of claim 1 wherein the A chain is ricin A chain, abrin toxin A chain, diphtheria toxin A chain, or Domain I of Pseudomonas exotoxin.
- 15 4. A nucleic acid molecule of claim 1 wherein the A chain is volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain or shiga toxin A chain.
5. A nucleic acid molecule of claim 1 wherein the B chain is ricin B chain, abrin toxin A chain, diphtheria toxin B chain, or Domain
20 II of Pseudomonas exotoxin.
6. A nucleic acid molecule of claim 1 wherein the B chain is volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain or shiga toxin B chain.
7. A nucleic acid molecule according to any one of claims 1
25 to 6 having a nucleic acid sequence selected from the group consisting of

the nucleic acid sequence of pAP301 as shown in Figure 1B; the nucleic acid sequence of pAP302 as shown in Figure 2B; the nucleic acid sequence of pAP303 as shown in Figure 3B; the nucleic acid sequence of pAP304 as shown in Figure 4B; the nucleic acid sequence of pAP305 as shown in Figure 5B; the nucleic acid sequence of pAP308 as shown in Figure 6B; the nucleic acid sequence of pAP309 as shown in Figure 7B; the nucleic acid sequence of pAP313 as shown in Figure 8B; the nucleic acid sequence of pAP314 as shown in Figure 9B; the nucleic acid sequence of pAP315 as shown in Figure 10B; the nucleic acid sequence of pAP316 as shown in Figure 11B; the nucleic acid sequence of pAP317 as shown in Figure 12B; the nucleic acid sequence of pAP318 as shown in Figure 13B; the nucleic acid sequence of pAP319 as shown in Figure 14B; the nucleic acid sequence of pAP320 as shown in Figure 15B; the nucleic acid sequence of pAP321 as shown in Figure 16B; the nucleic acid sequence of pAP322 as shown in Figure 17B; the nucleic acid sequence of pAP323 as shown in Figure 18B; the nucleic acid sequence of pAP324 as shown in Figure 19B; and the nucleic acid sequence of pAP325 as shown in Figure 20B.

8. A nucleic acid molecule according to any one of claims 1 to 7 wherein (c) the nucleotide sequence of the linker is selected from the group consisting of: the nucleic acid sequence of pAP301 as shown in Figure 1A; the nucleic acid sequence of pAP302 as shown in Figure 2A; the nucleic acid sequence of pAP303 as shown in Figure 3A; the nucleic acid sequence of pAP304 as shown in Figure 4A; the nucleic acid sequence of pAP305 as shown in Figure 5A; the nucleic acid sequence of pAP308 as shown in Figure 6A; the nucleic acid sequence of pAP309 as shown in Figure 7A; the nucleic acid sequence of pAP313 as shown in Figure 8A; the nucleic acid sequence of pAP314 as shown in Figure 9A; the nucleic acid sequence of pAP315 as shown in Figure 10A; the nucleic acid sequence of pAP316 as shown in Figure 11A; the nucleic acid sequence of pAP317 as shown in Figure 12A; the nucleic acid sequence

of pAP318 as shown in Figure 13A; the nucleic acid sequence of pAP319 as shown in Figure 14A; the nucleic acid sequence of pAP320 as shown in Figure 15A; the nucleic acid sequence of pAP321 as shown in Figure 16A; the nucleic acid sequence of pAP322 as shown in Figure 17A; the
5 nucleic acid sequence of pAP323 as shown in Figure 18A; the nucleic acid sequence of pAP324 as shown in Figure 19A; and the nucleic acid sequence of pAP325 as shown in Figure 20A.

9. A plasmid incorporating the nucleic acid molecule of any one of claims 1 to 8.

10 10. A baculovirus transfer vector incorporating the nucleic acid molecule according to any one of claims 1 to 8.

11. A recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker
15 sequence contains a cleavage recognition site for a disease-specific protease.

12. A recombinant protein of claim 11 wherein the A chain is ricin A chain, abrin toxin B chain, diphtheria toxin A chain, or Domain I of Pseudomonas exotoxin.

20 13. A recombinant protein of claim 11 wherein the A chain is volkensin toxin A chain, cholera toxin A chain, modeccin toxin A chain or shiga toxin A chain.

14. A recombinant protein of claim 11 wherein the B chain is ricin B chain, abrin toxin B chain, diphtheria toxin B chain, or Domain
25 II of Pseudomonas exotoxin.

15. A recombinant protein of claim 11 wherein the B chain is volkensin toxin B chain, cholera toxin B chain, modeccin toxin B chain or shiga toxin B chain.

16. A recombinant protein of claim 11 wherein the cleavage
5 recognition site is recognized by a cancer-associated protease selected from the group consisting of a matrix metalloproteinase and a urokinase-type plasminogen activator.

17. A recombinant protein of claim 11 wherein the linker
10 amino acid sequence is selected from the group consisting of: the amino acid sequence of PAP301 as shown in Figure 1C; the amino acid sequence of PAP302 as shown in Figure 2C; the amino acid sequence of PAP303 as shown in Figure 3C; the amino acid sequence of PAP304 as shown in Figure 4C; the amino acid sequence of PAP305 as shown in Figure 5C; the amino acid sequence of PAP308 as shown in Figure 6C;
15 the amino acid sequence of PAP309 as shown in Figure 7C; the amino acid sequence of PAP313 as shown in Figure 8C; the amino acid sequence of PAP314 as shown in Figure 9C; the amino acid sequence of PAP315 as shown in Figure 10C; the amino acid sequence of PAP316 as shown in Figure 11C; the amino acid sequence of PAP317 as shown in
20 Figure 12C; the amino acid sequence of PAP318 as shown in Figure 13C; the amino acid sequence of PAP319 as shown in Figure 14C; the amino acid sequence of PAP320 as shown in Figure 15C; the amino acid sequence of PAP321 as shown in Figure 16C; the amino acid sequence of PAP322 as shown in Figure 17C; the amino acid sequence of PAP323 as
25 shown in Figure 18C; the amino acid sequence of PAP324 as shown in Figure 19C; and the amino acid sequence of PAP325 as shown in Figure 20C.

18. A method of inhibiting or destroying cancer cells, which cells are associated with a protease specific to the cancer comprising the steps of:

- (a) preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin, and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for the protease;
- (b) introducing the nucleic acid into a host cell and expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a linker amino acid sequence;
- (c) suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient, and
- (d) contacting the cells with the recombinant protein.

19. A method of inhibiting or destroying cancer cells comprising contacting the cells with an effective amount a recombinant protein according to any one of claims 11 to 17.

20. A method of treating a cancer comprising administering an effective amount of a recombinant protein according to any one of claims 11 to 17 to an animal in need thereof.

21. A method of treating a cancer comprising administering an effective amount of a nucleic acid molecule according to any one of claims 1 to 7 to an animal in need thereof.

22. A process for preparing a pharmaceutical for treating cancer comprising the steps of :

- (a) preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain

of a ricin-like toxin, and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a cancer;

(b) introducing the nucleic acid into a host cell and
5 expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a linker amino acid sequence;

(c) suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

10 23. A pharmaceutical composition for treating cancer comprising a recombinant protein of any one of claims 11 to 17 and a pharmaceutically acceptable carrier, diluent or excipient.

24. A pharmaceutical composition for treating cancer comprising a nucleic acid molecule of any one of claims 1 to 7 and a
15 pharmaceutically acceptable carrier, diluent or excipient.

25. A purified and isolated nucleic acid molecule having a nucleic acid sequence selected from the group consisting of: the nucleic acid sequence of pAP301 as shown in Figure 1A; the nucleic acid sequence of pAP302 as shown in Figure 2A; the nucleic acid sequence of
20 pAP303 as shown in Figure 3A; the nucleic acid sequence of pAP304 as shown in Figure 4A; the nucleic acid sequence of pAP305 as shown in Figure 5A; the nucleic acid sequence of pAP308 as shown in Figure 6A; the nucleic acid sequence of pAP309 as shown in Figure 7A; the nucleic acid sequence of pAP313 as shown in Figure 8A; the nucleic acid
25 sequence of pAP314 as shown in Figure 9A; the nucleic acid sequence of pAP315 as shown in Figure 10A; the nucleic acid sequence of pAP316 as shown in Figure 11A; the nucleic acid sequence of pAP317 as shown in Figure 12A; the nucleic acid sequence of pAP318 as shown in Figure 13A; the nucleic acid sequence of pAP319 as shown in Figure 14A; the

nucleic acid sequence of pAP320 as shown in Figure 15A; the nucleic acid sequence of pAP321 as shown in Figure 16A; the nucleic acid sequence of pAP322 as shown in Figure 17A; the nucleic acid sequence of pAP323 as shown in Figure 18A; the nucleic acid sequence of pAP324 as shown in Figure 19A; and the nucleic acid sequence of pAP325 as shown in Figure 20A.

26. A linker protein having an amino acid sequence selected from the group consisting of: the amino acid sequence of PAP301 as shown in Figure 1C; the amino acid sequence of PAP302 as shown in Figure 2C; the amino acid sequence of PAP303 as shown in Figure 3C; the amino acid sequence of PAP304 as shown in Figure 4C; the amino acid sequence of PAP305 as shown in Figure 5C; the amino acid sequence of PAP308 as shown in Figure 6C; the amino acid sequence of PAP309 as shown in Figure 7C; the amino acid sequence of PAP313 as shown in Figure 8C; the amino acid sequence of PAP314 as shown in Figure 9C; the amino acid sequence of PAP315 as shown in Figure 10C; the amino acid sequence of PAP316 as shown in Figure 11C; the amino acid sequence of PAP317 as shown in Figure 12C; the amino acid sequence of PAP318 as shown in Figure 13C; the amino acid sequence of PAP319 as shown in Figure 14C; the amino acid sequence of PAP320 as shown in Figure 15C; the amino acid sequence of PAP321 as shown in Figure 16C; the amino acid sequence of PAP322 as shown in Figure 17C; the amino acid sequence of PAP323 as shown in Figure 18C; the amino acid sequence of PAP324 as shown in Figure 19C; and the amino acid sequence of PAP325 as shown in Figure 20C.

ABSTRACT OF THE DISCLOSURE

The present invention provides a protein having chain of a ricin-like toxin, a B chain of a ricin-like toxin and a novel heterologous linker amino acid sequence, linking the A and B chains.

- 5 The linker sequence contains a cleavage recognition site for a cancer specific protease. The invention also relates to a nucleic acid molecule encoding the protein and to expression vectors incorporating the nucleic acid molecule. Also provided is a method of inhibiting or destroying cancer cells utilizing the nucleic acid molecules and proteins
- 10 of the invention and pharmaceutical compositions for treating human cancer.

FIGURE 1A

Sequence of pAP301 (MMP-9) Linker Region

WT preprorincin linker

```

                    primer 301-3'
                    5' - ATGTGGGACACGAAATTTTAATGCTGAT -3'
                        * *** **** *
-CTCATGGTGTATAGATGGCGCACCTCCACCATCGTCACAGTTTCTTTGCTTATA|AGGCCAGTGGTACCAAAATTTTAATGCTGATGTTGTATGGATCCTGAGCCC-
-GAGTACCAACATATCTACGGGTGGAGGTGGTAGCAGTGTCAAAAGAAACGAATAT|TCCGGTCAACCATGGTTTAAATTAACGACTACAAACATACCTAGGACTCGGG-
                        ** *** ***
3' - GGTGGTAGCAGTGTCAAAACCAAGGAGAACCG -5'
                    primer 301-5'

```

1) PCR mutagenesis

2) Ligate with pVL1393

```

pAP301 linker
(MMP-9 variant)
GCACCTCCACCATCGTCACAGTTTGTCTCTTGGC|ATGTGGGACACGAAATTTTAATGCTGATGTT
CGTGGAGGTGGTAGCAGTGTCAAAACCAAGGAGAACCG|TACACCCCTGTTGCTTTAAATTAACGACTACAA

```

Note: Nucleotides in bold are found within the preprorincin linker region. The '.' symbol within the linker designate deleted nucleotides.

FIGURE 1B (P1)

Sequence of pAP301 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAACGTAGGTTTACTAAAGTCTTCGTCGTTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

FIGURE 1B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGGTCCCTCTTGGCATGTGGGGACAACGAAATTTTAATGC
AGCAGTGTCAAACCAGGAGAACCGTACACCCCTGTTGCTTTAAAATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCAAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACCTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

FIGURE 1B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTTAAATTTGTATAGTGGGTTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCTGTGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: pAP301

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

(c) $\frac{d}{dt} \left(\frac{\partial L}{\partial \dot{x}} \right) = \frac{\partial L}{\partial x}$

where L is the Lagrangian function.

Wild type ricin linker: A chain- C A P P P S S Q F S L L I R P V V P N F N A D V C M D P E - B chain

PAP301 (MMP-9) linker: A chain- C A P P P S S Q F G P L G M W G Q R N F N A D V C M D P E - B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

FIGURE 2A

Sequence of pAF302 (MMP-9) Linker Region

WT preproricin linker

```

                    primer 302-3'
5'- GGGCAG-----TGTATGGATCCTGAGCCC -3'
      * ***
-CTCATGGGTATAGATGCGCACCTCCACCATCGTCACAGTTTCTTTGCTTATAAGGCCA | GTGGTACCAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
-GAGTACCA CATATCTACGCGTGGAGGTGGTAGCAGTGTCAAAGAAACGATATTCCGGT | CACCATGGTTTAAATTACGACTACAAACATACCTAGGACTCGGG-
      ** **** ***
3'- AGCAGTGTCAAAGAGGCGTTCCTTAAACGT -5'
      primer 302-5'

```

1) PCR mutagenesis

2) Ligate with pVL1393

```

pAF302 linker
(MMP-9 variant)
GCACCTCCACCATCGTCACAGTTTCTCCGCAAGGAATTGCA | GGGCAG
CGTGGAGGTGGTAGCAGTGTCAAAGAGGCGTTCCTTAAACGT | CCCGTC

```

Note: Nucleotides in bold are found within the preproricin linker region. The '.' symbol within the linker designate deleted nucleotides.

FIGURE 2B (P1)

Sequence of pAP302 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCACCAGAAAGTGTAATC
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAATAAGTTTAA
451 CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCCTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGAGCAGCAAG
GACCGAGCAAGGAAATATTAACGTTAGGTTTACTAAAGTCTTCGTCGTTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

FIGURE 2B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTCCGCAAGGAATTGCAGGGCAG-----
AGCAGTGTCAAAGAGGGCGTTCCTTAACGTCCCGTC-----

951 -----TGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGAAATG
-----ACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTACGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTATACCTATCTCCTGACATCGTCACTTT

FIGURE 2B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCCTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1834.

Sequence name: pAP302

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

[illegible]

Note: Amino acids in bold are found within the preproricin linker region. The ‘-’ symbol within the linker designate deleted amino acids.

Note: Amino acids in bold are found within the preproricin linker region. The ‘-’ symbol within the linker designate deleted amino acids.

FIGURE 3A

Sequence of pAP303 (MMP-9) Linker Region

WT preporicrin linker

primer 303-3'
 5' - GGGCAGCGAAATTTTAATGCTGAT -3'
 * * * * *
 -CTCATGGGTATAGATGCGCACCTCCACCATCGTCACAGTTTCTTTGCTTATAAGGCCA | GTGGTACCAAAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
 -GAGTACCACATATCTACCGCGTGGAGGTGGTAGCAGTGTCAAAAGAAACGAATATTCGGGT | CACCATGGTTTAAATTTACGACTACAAACATACCTAGGACTCGGG-
 ** * * * * *
 3' -GAGTACCACATATCTACG-----AGAGGCGTTCCTTAACGT -5'
 primer 303-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP303 linker
 (MMP-9 variant)
 TCTCCGCAAGGAATTGCA | GGGCAGCGAAATTTTAATGCTGATGTT
 AGAGGCGTTCCTTAACGT | CCGTGGCTTTAAATTTACGACTACAA

Note: Nucleotides in bold are found within the preporicrin linker region. The '-' symbol within the linker designate deleted nucleotides.

FIGURE 3B (P1)

Sequence of pAP303 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGCG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA				
	ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACCTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTACGCGTGCTCTTAATCCATGTTGGCCT				

FIGURE 3B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGC-----
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACG-----

901 -----TCTCCGCAAGGAATTGCAGGGCAGCGAAATTTTAATGC
-----AGAGGCGTTCCTTAACGTCCCGTCGCTTTAAATACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCACAAACGGAAACGCAATA
CAGATACACAACATAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

FIGURE 3B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAAACATTTAAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1831.

Sequence name: pAP303

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

Amino acid sequence Comparison of Mutant Preprotrisin Linker Region of PAP303 (MMP-9) to Wild Type

Wild type ricin linker: A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E - B chain

PAP303 (MMP-9) linker: A chain- C - - - - - S P Q G I A G Q R N F N A D V C M D P E - B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

FIGURE 4A

Sequence of pAP304 (MMP-9) Linker Region

WT preprorin linker

5' - GGGCAG - primer 304-3'
 * * *
 -CTCATGGTGTATAGATGGCCACCTCCACCATCGTCACAGTTTCTTTGCTTATAAGGCCA | GTGGTACCAAAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
 -GAGTACCACATATCTACGGGTGGAGGTGGTAGCAGTGTCAAAGAAACGAATATTCGGT | CACCATGGTTTAAATACGACTACAAACATACCTAGGACTCGGG-
 ** **** *
 3' -GAGTACCACATATCTACG-----AGAGGCGTTCCTTAACGT -5'
 primer 304-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP304 linker

(MMP-9 variant)

TCTCCGCAAGGAATTGCA | GGGCAG

AGAGGCGTTCCTTAACGT | CCGGTC

Note: Nucleotides in bold are found within the preprorin linker region. The '.' symbol within the linker designate deleted nucleotides.

FIGURE 4B (P1)

Sequence of pAP304 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTTCGCGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCACTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTCTGTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTA				
451	CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG				
501	TGGTAATCTGAGAGAAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

FIGURE 4B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGC-----
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACG-----

901 -----TCTCCGCAAGGAATTGCAGGGCAG-----
-----AGAGGCGTTCCTTAACGTCCCGTC-----

951 -----TGTATGGATCCTGAGCCCATAGTGCATCGTAGGTCGAAATG
-----ACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATAACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTCACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

FIGURE 4B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1810.

Sequence name: pAP304

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

[illegible]

Amino acid sequence Comparison of Mutant Preprotricin Linker Region of PAP304 (MMP-9) to Wild Type

Wild type ricin linker: A chain- C A P P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain

PAP304 (MMP-9) linker: A chain- C - - - - S P Q G I A G Q - - - - C M D P E -B chain

Note: Amino acids in bold are found within the preprotricin linker region. The ‘.’ symbol within the linker designate deleted amino acids.

FIGURE 5A

Sequence of pAP305 (MMP-9) Linker Region

WT preprorincin linker

```

                    primer 305-3'
                    5' - GGGCAG-----TGATGGATCCTGAGCCC -3'
                        * ***
-CTCATGGGTATAGATGGCGCACCTCCACCATCGTCCACAGTTTCTTTGCTTATAAGCCA | GTGGTACCAAAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
-GAGTACCATATCTACGCGTGGAGGTGGTAGCAGTGTCAAAAGGAACGAATATTCGGGT | CACCATGGTTTAAATTTACGACTACAAACATACCTAGGACTCGGG-
                        ** **** ***
3' - TCTACGCGTGGAGGTGGT-----AGAGGCGTTCCTTAACGT -5'
    primer 305-5'

```

1) PCR mutagenesis

2) Ligate with pVL1393

```

pAP 305 linker
(MMP-9 variant)
GCACCTCCACCATCTCCGCAAGGAATTGCA | GGGCAG
CGTGGAGGTGGTAGAGGCGTTCCTTAACGT | CCCGTC

```

Note: Nucleotides in bold are found within the preprorincin linker region. The '-' symbol within the linker designate deleted nucleotides.

FIGURE 5B (P1)

Sequence of pAP305 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC
101 AGGATAACAACATATTCCTCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTCCGCG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACTGAGCTGATGTGAGACATGAAATACCACTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTAGTTGAACTCTCA
TGTCTCAACCAACCGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCAGCAGCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTGA
451 CGATATACATTTCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTGTAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAACGTAGGTTTACTAAAGTCTTCGTCGTTG
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

FIGURE 5B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 -----TCTCCGCAAGGAATTGCAGGGCAG-----
-----AGAGGCGTTCCTTAACGTCCCGTC-----

951 -----TGTATGGATCCTGAGCCATAGTGCGTATCGTAGGTCGAAATG
-----ACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACAGTTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTTACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

FIGURE 5B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1822.

Sequence name: pAP305

Note: Nucleotides in bold are found within the mutant preprorycin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 5C

Amino acid sequence Comparison of Mutant Preprotricin Linker Region of PAP305 (MMP-9) to Wild Type

Wild type ricin linker: A chain- C A P P P S S Q F S L L I R P V V P N F N A D V C M D P E - B chain

PAP305 (MMP-9) linker: A chain- C A P P P - - - S P Q G I A G Q - - - - - C M D P E - B chain

Note: Amino acids in bold are found within the preproricin linker region. The ‘.’ symbol within the linker designate deleted amino acids.

FIGURE 6A

Sequence of pAP308 (MMP-9) Linker Region

WT preprorin linker

5' - ATGTGGGGACAA - - - - - primer 308 - 3'

* * * * *

CTCATGGTGTATAGATCGCACCTCCACCATCGTCACAGTTTCTTGTCTTATA | AGGCCAGTGGTACCAAAATTTTAAATGCTGATGTTTGTATGGATCCTGAGCCCATAGTGGGTATCGTA -
 -GAGTACCACATATCTACGCGTGGAGGTGGTAGCAGTGTCAAAAGAAACGAATAT | TCCGGTCACCATGGTTTAAATTTACGACTACAAACATACCTAGGACTCTGGGTATCAGCGCATAGCAT -
 * * * * *

3' - TCTACGCGTGGAGGTGGT - - - - - primer 308 - 5'

* * * * *

TTGTGGTGGCGGAGGGCCCATAGTGGTATCGTA - 3'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP 308 linker (MMP-9 variant)

GCACCTCCACACAGTCTCTTGGC | ATGTGGGGACAA
 CGTGGAGGTGGTCCAGGAGAACCG | TACACCCCTGTT

Note: Nucleotides in bold are found within the preprorin linker region. The '-' symbol within the linker designate deleted nucleotides.

FIGURE 6B (P1)

Sequence of pAP308 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTGA
451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTCC
651 ATCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

FIGURE 6B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 -----GGTCCTCTTGGCATGTGGGGACAA-----
-----CCAGGAGAACCGTACACCCCTGTT-----

951 -----TGTGGTGGCGGAGGGCCCATAGTGCCTATCGTAGGTCGAAATG
-----ACACCACCGCCTCCCGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTACAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATAACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAAAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

FIGURE 6B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTAGAT
AGTTCTTACTACCTTGGTAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCTGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1822.

Sequence name: pAP308

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 6C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP308 (MMP-9) to Wild Type

wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP308 (MMP-9) linker:	A chain- C A P P - - - G P L G M W G Q - - - - C G G G G -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

FIGURE 7A

Sequence of pAP309 (MMP-9) Linker Region

WT preprorincin linker

primer 309-3'
 5' - TTTAATGCTGATGTTTGTGGTGGGAGGGCCCATAGTGGTATCGTA
 *** ***** *
 -CTCATGGTGATAGATGGCGCACCTCCACCATCGTCACAGTTTCTTTGCTTATAAGGCCAGTGGTACCAAAT | TTTAATGCTGATGTTTGTATGGATCCTGAGCCCATAGTGGTATCGTA -
 -GAGTACCACATATCTACCGGTGGAGGTGGTAGCAGTGTCAAAAGAAACGAATATTCGGGTCAACCATGTTTA | AAATTACGACTACAAACATACCTAGGACTCGGGTATCAGCGCATAGCAT -
 ** *** *** ***** *
 3' - GGTTGAGCAGTGTCAAAACAGAGAACCGTACACCCCTGTTGCTTTA -5'
 primer 309-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP309 linker (MMP-9 variant)

GCACCTCCACCATCGTCACAGTTTGGTCCTCTTGGCATGTGGGGACACGAAAT | TTTAATGCTGATGTT
 CGTGGAGGTGGTAGCAGTGTCAAAACAGAGAACCGTACACCCCTGTTGCTTTA | AAATTACGACTACAA

Note: Nucleotides in bold are found within the preprorincin linker region. The '-' symbol within the linker designate deleted amino acids.

FIGURE 7B (P1)

Sequence of pAP309 insert

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTCGCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTCAAGTTTGA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAT
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAACGTAGGTTTACTAAAGTCTTCGTCGTTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGSCCT

FIGURE 7B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGGTCCTCTTGGCATGTGGGGACAACGAAATTTTAATGC
AGCAGTGTCAAACCAGGAGAACCGTACACCCCTGTTGCTTTAAATTACG

951 TGATGTTTGTGGTGGCGGAGGGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACACCACCGCTCCCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACCTATCTCCTGACATCGTCACTTT

FIGURE 7B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: pAP309

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 7C

Amino acid sequence Comparison of Mutant Preprotrisin Linker Region of PAP309 (MMP-9) to Wild Type

Wild type ricin linker: A chain- C A P P P S S Q F S L L I R P V V P N F N A D V C M D P E - B chain

PAP-309 (MMP-9) linker: A chain- C A P P P S S Q F G P L G M W G Q R N F N A D V C G G G G - B chain

Note: Amino acids in bold are found within the preproinsulin linker region. The '-' symbol within the linker designate deleted amino acids.

[illegible]

WT preprorin linker

primer 313-3'-----TGTATGGATCCTGAG -3'
 5'/- GTAGTCGGCGGG-----* ***** *
 -CTCANGGTGATAGATGGCGCACCTCCACCATCGTCACAGTTTCTTTGCTT | ATTAGGCCAGTGGTACCAAAATTTAAATGCTGATGTTGTATGGATCCTGAGCCCC-
 -GAGTACCACATATCTACGCGGTGGAGGTGGTAGCAGTGTCAAAAGAAACGAA | TATTCGGGTCAACCATGGTTTAAATACGACTACAAACATACCTAGGACTCGGG-
 * ***** **
 3' -TACCACATATCTACG-----GGTCCTGCT -5'
 primer 313-5'

- 1) PCR mutagenesis
- 2) Ligate with pVL1393

**pAP313 linker
(UPA variant)**
CCAGGACGA | GTAGTCGGCGGG
GGTCTGCT | CATCAGCCGCCC

Note: Nucleotides in bold are found within the preprorin linker region. The ‘.’ symbol within the linker designate deleted nucleotides.

FIGURE 8B (P1)

Sequence of pAP313 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA				
	ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTTCGCCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC				
	GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCTGTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

FIGURE 8B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGC-----
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACG-----

901 -----CCAGGACGAGTAGTCGGCGGG-----
-----GGTCCTGCTCATCAGCCGCC-----

951 -----TGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
-----ACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCCCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

FIGURE 8B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAAACATTGACTTTCTGTGCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1807.

Sequence name: pAP313

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 8C

Amino acid sequence Comparison of Mutant Preprorenin Linker Region of PAP313 (UPA) to Wild Type

Wild type ricin linker: A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E - B chain

PAP313 (UPA) linker: A chain- C - - - - - P G R V V G G - - - - - C M D P E - B chain

Note: Amino acids in bold are found within the preprocrinin linker region. The '-' symbol within the linker designate deleted amino acids.

FIGURE 9A

Sequence of pAP314 (UPA) Linker Region

WT preprorin linker

```

                    primer 314-3'
5' - GTAGTCGGCGGG-----GGAGCGGGGGTTGTATGGATCCTGAG -3'
      * ***** *          *** ** ** *
-CTCATGGTGTATAGATGGCGCACCTCCACCATCGTCACAGTTTCTTTGCTT|ATAAGGCCAGTGGTACCAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
-GAGTACCAATATCTACGGGTGAGGTGGTAGCAGTGTCAAAAGAAACGAA|TATTCGGTCCACCATGGTTTAAATACCACTACAACATACCTAGGACTCGGG-
      * ***** *          * **** *
3' -TACCACATATCTACGCTCCGCCCCCA-----GGTCCTGCT -5'
                    primer 314-5'

```

1) PCR mutagenesis

2) Ligate with pVL1393

pAP314 linker (UPA variant)

```

GGAGCGGGGGTCCAGGACGA|GTAGTCGGCGGGGGAGGCGGGGGT
CCTCGCCCCCAGGTCCTGCT|CATCAGCGCCCCCTCCGCCCCCA

```

Note: Nucleotides in bold are found within the preprorin linker region. The '.' symbol within the linker designate deleted nucleotides.

FIGURE 9B (P1)

Sequence of pAP314 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC
101 AGGATAACAACATATTCCTCCAAACAATACCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACTGAGCTGATGTGAGACATGAAATACCAGTGTTCGCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTAGTTGAACTCTCA
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTGA
451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACTTGTGTAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCTGTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

FIGURE 9B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGCGGGGGT
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCGCCCCCA

901 -----CCAGGACGAGTAGTCGGCGGG-----GGAGG
-----GGTCCTGCTCATCAGCCGCC-----CCTCC

951 CGGGGGTTGTATGGATCCTGAGCCATAGTGCGTATCGTAGGTCGAAATG
GCCCCAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCACAAACGGAAACGCAATA
CAGATACAACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGAAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTT

FIGURE 9B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAA TGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCA GTGTGTGTCTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGT CACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1831.

Sequence name: pAP314

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

Amino acid sequence Comparison of Mutant Preprotricin Linker Region of PAP314 (UPA) to Wild Type

Wild type ricin linker: A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E - B chain

PAP314 (UPA) linker: A chain- C G G G G - - - - P G R V V G G - - - - G G G G C M D P E - B chain

Note: Amino acids in bold are found within the preproinsulin linker region. The '-' symbol within the linker designate deleted amino acids.

FIGURE 10A

Sequence of pAP315 (UPA) Linker Region

WT prepororicin linker

```

                    primer 315-3'
5' - CCAGGACGAGTAGTAGTCGGCGGG-----TGTATGGATCCTGAG -3'
    ** * * * *
-CTCATGGTGTATAGATGCGCACCTCCACCATCGTCACAGTTTCTTTGCTT|ATAAGGCCAGTGGTACCAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
-GAGTACCCACATATCTACCGTGGAGGTGGTAGCAGTGTCAAAGAAAGGAA|TATTCGGGTACCATGGTTTAAATTACGACTACAAACATACCTAGGACTCGGG-
    * * * * *
3' -TACCACATATCTACG-----GGTCCTGCTCATCAGCGGCC -5'
                    primer 315-5'

```

1) PCR mutagenesis

2) Ligate with pVL1393

pAP315 linker (UPA variant)

```

CCAGGACGAGTAGTCGGCGGG|CCAGGACGAGTAGTCGGCGGG
GGTCCTGCTCATCAGCGGCC|GGTCCTGCTCATCAGCGGCC

```

Note: Nucleotides in bold are found within the prepororicin linker region. The '-' symbol within the linker designate deleted nucleotides.

FIGURE 10B (P1)

Sequence of pAP315 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC				
101	AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG				
	CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC				
	GCTATATGTAAGCGGAAACCACCATTAACTATCTGAACTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCCTTTATAATTTGCATCCAAATGATTTTCAAGAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

FIGURE 10B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGC-----
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACG-----

901 CCAGGACGAGTAGTCGGCGGGCCAGGACGAGTAGTCGGCGGG-----
GGTCCTGCTCATCAGCCGCCCGGTCCTGCTCATCAGCCGCC-----

951 -----TGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGCAAATG
-----ACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTCAAGTTATGTCTACGTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTACATCCTATCTCCTGACATCGTCACTTT

FIGURE 10B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTGGTGTAGAT
AGTTCCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCACTGTGTGTCTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1828.

Sequence name: pAP315

Note: Nucleotides in bold are found within the mutant preprorcin linker region. The '-' symbol within the linker region designate deleted nucleotides.

[illegible]

Wild type ricin linker: A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E - B chain

PAP315 (UPA) linker: A chain- C - - - P G R V V G G P G R V V G G - - - C M D P E - B chain

Note: Amino acids in bold are found within the preproricin linker region. The ‘-’ symbol within the linker designate deleted amino acids.

FIGURE 11A

Sequence of pAP316 (MMP-9) Linker Region

WT preporicrin linker

primer 316-3'
 5' - ATTGCAGGCGAGGAGGGGTAGTAGCGGCGGGGGATGTATGGATCCTGAG -3'
 ***** * ***** ** * ** ** **
 -CTCATGGTGTATAGATGGCGCACCTCCACCATCGTCAAGTTTCTTTGCTT|ATAAGGCCAGTGGGTACCAATTTTAATGCTGATGTTGTATGGATCCTGAGCCCC-
 -GAGTACCACATATCTACCGGTGGAGGTGTAGCAGTGTCAAAGAAACGAA|TATTCCGGTCCACCATGGTTTAAATTAACGACTACAAACATACCTAGGACTCGGG-
 * ***** ** * *****
 3' -TACCACATATCTACGCCTCCGCCCTGAGGTCCGCCCCCAGGGGTTCT -5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP316 linker (MMP-9 variant)

GGAGCGGGGACTCCAGCGGGGGTCCGCAAGGA|ATTGCAGGGCAGGAGGGGGTAGTAGCGGCGGGGA
 CCTCGCCCCCTGAGGTGCGCCCCCAGGCGTTCTT|TAACGTCCCGTCCCTCCCCCATCATCGCGCCCCCT

Note: Nucleotides in bold are found within the preporicrin linker region. The '-' symbol within the linker designate deleted nucleotides.

FIGURE 11B (P1)

Sequence of pAP316 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTACATTAG				
	CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC				
101	AGGATAACAACATATTCCTCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTTCGCGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACTGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTTCTGTACATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA				
	ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTACAAGTTT				
451	CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCTTC				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

FIGURE 11B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGCGGGGGT
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCGCCCTGA

901 GGAGGCGGGGGTCCGCAAGGAATTGCAGGGCAGGGAGGGGGTAGTAGCGG
GGTCCGCCCCCAGGCGTTCTTAACGTCCCGTCCCTCCCCCATCATCGCC

951 CGGGGGATGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
GCCCCCTACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

FIGURE 11B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTGTACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTCTTACTACCTTGGTAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: pAP316

Note: Nucleotides in bold are found within the mutant preprorcin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 11C

Amino acid sequence Comparison of Mutant Preprorin Linker Region of PAP316 (MMP-9) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP316 (MMP-9) linker:	A chain- C G G G S S G G G P Q G I A G Q G G G S S G G G C M D P E -B chain

Note: Amino acids in bold are found within the preprorin linker region. The '-' symbol within the linker designate deleted amino acids.

FIGURE 12A

Sequence of pAP317 (MMP-9) Linker Region

WT preporicrin linker

primer 317-3'
 5' - TATCCAATAGTGCAAAATTTTACAGCTGATGTTGTATG -3'
 *** * * * *
 -CTCATGGTGATAGATGCGCACCTCCACCATCGTCACAGTTTCTTTGCTTATA|AGGCAGTGGTACCAAAATTTTAAATGCTGATGTTGTATGGATCCTGAGCCCC-
 -GAGTACCACATATCTACGCGTGAGGTGGTAGCAGTGTCAAAGAAAGCAATAT|TCCGGTCACCATGGTTTAAATTTACGACTACAAACATACCTAGGACTCGGG-
 ** * * * *
 3' - GGTGGTAGCAGTGTCAAACAAAGCGTCTTG -5'
 primer 317-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP317 linker (MMP-9 variant)

GCACCTCCACCATCGTCACAGTTTGTTCGCAGAAC|TATCCAATAGTGCAAAATTTTACAGCTGATGTT
 CGTGGAGGTGGTAGCAGTGTCAAACAAAGCGTCTTG|ATAGGTTATCACGTTTTTAAATTTGCGACTACAA

Note: Nucleotides in bold are found within the preporicrin linker region. The '.' symbol within the linker designate deleted nucleotides.

FIGURE 12B (P1)

Sequence of pAP317 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC
101 AGGATAACAACATATTTCCCAAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACAACCTGGAGCTGATGTGAGACATGAAATACCAGTGTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA
451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGCAGCAAG
GACCGAGCAAGGAAATATTAACGTAGGTTTACTAAAGTCTTCGTCGTTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

FIGURE 12B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTGTTCGCGAGAACTATCCAATAGTGCAAAATTTTACAGC
AGCAGTGTCAAACAAAGCGTCTTGATAGGTTATCACGTTTAAATGTGC

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTAACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACCAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATCTCTCCTGACATCGTCACTTT

FIGURE 12B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: pAP317

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 12C

Amino acid sequence Comparison of Mutant Preprorin Linker Region of PAP317 (MMP-9) to Wild Type

Wild type ricin linker: A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain

PAP317 (MMP-9) linker: A chain- C A P P S S Q F V S Q N Y P I V Q N F T A D V C M D P E -B chain

Note: Amino acids in bold are found within the preprorin linker region. The ‘.’ symbol within the linker designate deleted amino acids.

FIGURE 13A

Sequence of pAP318 (MMP-9) Linker Region

WT preprorin linker

primer 318-3'
 5'- ATTGCAGGGCAGGATGAAGAGGATGCTGTTGTATG -3'
 **** * *****
 -CTCATGGTGTATAGATGGGCACCTCCACCATCGTCACAGTTTCTTTGCTTATA | AGSCCAGTGGTACCAAAATTTAAATGCTGATGTTTGTATGGATCCTGAGCCC-
 -GAGTACCACATATCTACGGGTGGAGTGGTAGCAGTGTCAAAAGAAACGAATAT | TCCGGTCACCATGGTTTAAATACGACTACAAACATACCTAGGACTCGGG-
 ***** ** ****
 3'- GGAGTGGTAGCAGTCTCTCAAGAGGCGTTCT -5'
 primer 318-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP318 linker (MMP-9 variant)

GCACCTCCACCATCGTCAGGAGTTCTCCGCAAGGA | ATTGCAGGGCAGGATGAAGAGGATGCTGATGTT
 CGTGGAGTGGTAGCAGTCTCTCAAGAGGCGTTCT | TAACGTCCCGTCTTCTCTCTAGACTACAA

Note: Nucleotides in bold are found within the preprorin linker region. The '.' symbol within the linker designate deleted nucleotides.

FIGURE 13B (P1)

Sequence of pAP318 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAACT
101 AGGATAACAACATATTTCCCAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTGTTATGGGTAAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAAACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTGCGAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA
451 CGATATACATTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAAGTTGTTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAACCGTAGGTTTACTAAAGTCTTCGTCGTTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

FIGURE 13B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCGGAGGTTCTCCGCAAGGAATTGCAGGGCAGGATGAAGAGGAATGC
AGCAGCCTCCAAGAGGCGTTCCTTAACGTCCCGTCTACTTCTCCTTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGAAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

FIGURE 13B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTTAAATTTGTATAGTGGGTTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCACTGTGTGTGTCTCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: pAP318

Note: Nucleotides in bold are found within the mutant preprorcin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 13C

Amino acid sequence Comparison of Mutant Preprorin Linker Region of PAP318 (MMP-9) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP318 (MMP-9) linker:	A chain- C A P P S S G G S P Q G I A G Q D E E D A D V C M D P E -B chain

Note: Amino acids in bold are found within the preprorin linker region. The '-' symbol within the linker designate deleted amino acids.

FIGURE 14A

Sequence of pAP319 (MMP-9) Linker Region

WT preprorin linker

primer 319-3'
5' - AATTATGATGAAGAGGATGCTGATGTTTGTATG -3'

-CTCATGGTGTATAGATGGCACCCTCCACCATCGTCACAGTTTCTTCTTATAGGCCA|GTGGTACCAAAATTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
-GAGTACCACATATCTACGCGTGGAGGTGGTAGCAGTGTCAAAGAAAGGAATATTCGGGT|CACCATGCTTTAAATACGACTACAAACATACCTAGGACTCGGG-
***** * ** **
3' - GGAGGTGGTAGCAGTCTCCAGTCCACCAAGTTAAGTC -5'
primer 319-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP319 linker (MMP-9 variant)

CCTCCACCATCGTCAGGAGGTGAGTGGTCAATTGCAG|AATTATGATGAAGAGGATGCTGATGTT
GGAGGTGGTAGCAGTCTCCAGTCCACCAAGTTAAGTC|TTAATACTACTTCTCCTAGGACTACAA

Note: Nucleotides in bold are found within the preprorin linker region. The '-' symbol within the linker designate deleted nucleotides.

FIGURE 14B (P1)

Sequence of pAP319 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTGCGGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACTGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA
451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

FIGURE 14B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCAGGAGGTGAGGTGGTTCAATTGCAGAATTATGATGAAGAGGATGC
AGCAGTCCTCCAGTCCACCAAGTTAACGTCTTAATACTACTTCTCCTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCATATCGTAGGTGAAAATG
ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTGAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTTACAACCATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

FIGURE 14B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTGTACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTTAAATTTGTATAGTGGGTTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1855.

Sequence name: pAP319

Note: Nucleotides in bold are found within the mutant preprorcin linker region. The '-' symbol within the linker region designate deleted nucleotides.

Amino acid sequence Comparison of Mutant Preprotrisin Linker Region of PAP319 (MMP-9) to Wild Type

Wild type ricin linker: A chain- C A P P P S S Q F S L L I R P V V P N F N A D V C M D P E - B chain

PAP319 (WMP-9) linker: A chain- C A P P P S S G G Q V V Q L Q N Y D E E D A D V C M D P E - B chain

Note: Amino acids in bold are found within the preproinsulin linker region. The ‘-’ symbol within the linker designate deleted amino acids.

FIGURE 15A

Sequence of pAP320 (UPA) Linker Region

WT preporicin linker

```

                    primer 320-3'
5' - GTAGTCGGCGGG-----GGGGGAGGCTGTATGGATCCTGAG -3'
      * ***** *          ** ** **
-CTCATGGGTATAGATGGGCACTCCACCATCGTCAAGTTTCTTTGCTT | ATAAGCCAGTGGTACCAATTTTAAATGCTGATGTTTGTATGGATCCTGAGCCC-
-GAGTACCACATATCTACCGGTGGAGGTGGTAGCAGTGTCAAAAGAAACGAA | TATTCGGTCCACCATGGTTTAAATTACGACTACAAACATACCTAGGACTCGGG-
      * ***** *          ** ** **
3' -TACCACATATCTACGCCTCGGCT-----GGTCCTGCT -5'
                    primer 320-5'

```

1) PCR mutagenesis

2) Ligate with pVL1393

pAP320 linker (UPA variant)

```

GGAGGGCGGACCAGGACGA | GTAGTCGGCGGGGGGGGAGGC
CCTCCGCTGCTGCTGCT | CATCAGCCGCCCCCCCCCTCCG

```

Note: Nucleotides in bold are found within the preporicin linker region. The '-' symbol within the linker designate deleted nucleotides.

FIGURE 15B (P1)

Sequence of pAP320 insert

	10	20	30	40	50
1	GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT				
	CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA				
51	GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG				
	CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC				
101	AGGATAACAACATATTTCCCAAACAATACCCAATTATAAACTTTACCACA				
	TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT				
151	GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTGCGGG				
	CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC				
201	TCGTTTAACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTGCCAA				
	AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT				
251	ACAGAGTTGGTTTGCCTATAAAACCAACGGTTTATTTTAGTTGAACTCTCA				
	TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT				
301	AATCATGCAGAGCTTCTGTTCATTAGCGCTGGATGTCACCAATGCATA				
	TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT				
351	TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA				
	ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT				
401	ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT				
	TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA				
451	CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC				
	GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTTGTTGAACG				
501	TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG				
	ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC				
551	CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC				
	GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA				
601	CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTTCAAGAGCAGCAAG				
	GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT				
651	ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA				
	TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT				

FIGURE 15B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGCGGA---
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCGCCT---

901 -----CCAGGACGAGTAGTCGGCGGG-----GG
-----GGTCCTGCTCATCAGCCGCC-----CC

951 GGGAGGCTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
CCCTCCGACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

FIGURE 15B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1825.

Sequence name: pAP320

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 15C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP320 (UPA) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP320 (UPA) linker:	A chain- C G G G - - - - P G R V V G G - - - - G G G C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

[illegible]

WT preproricin linker

[illegible]

- 1) PCR mutagenesis
- 2) Ligate with pVL1393

pAP321 linker
(UPA variant)
GGAGGCCAGGACGA | GTAGTGGGGGGGAGGC
CTTCGGGTCTGCT | CATCAGCCGCCCTCTCG

Note: Nucleotides in bold are found within the preprorin linker region. The ‘-’ symbol within the linker designate deleted nucleotides.

FIGURE 16B (P1)

Sequence of pAP321 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAAATC
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCACGGTGACACGTTTCGATGTGTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACTGAGCTGATGTGAGACATGAAATACCAGTGTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTGA
451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTGTAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAT
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAACGTAGGTTTACTAAAGTCTTCGTCGTTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

FIGURE 16B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGC-----
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCG-----

901 -----CCAGGACGAGTAGTCGGCGGG-----
-----GGTCCTGCTCATCAGCCGCC-----

951 -GGAGGCTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTGAAAATG
-CCTCCGACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTGAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTCATACCTATCTCCTGACATCGTCACTTT

FIGURE 16B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTGTACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1819.

Sequence name: pAP321

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

[illegible]

Note: Amino acids in bold are found within the prepronic linker region. The ‘-’ symbol within the linker designate deleted amino acids.

Note: Amino acids in bold are found within the preproinsin linker region. The ‘-’ symbol within the linker designate deleted amino acids.

[illegible]

WT preprorin linker

1) PCR mutagenesis

2) Ligate with pVL1393

pAP322 linker
(UPA variant)
GGACCAGGACGA | GTAGTCGGCGGGGGC
CCTGGTCTCTGCT | CATCAGCCGCCCCCCG

Note: Nucleotides in bold are found within the preprorion linker region. The ‘.’ symbol within the linker designate deleted nucleotides.

FIGURE 17B (P1)

Sequence of pAP322 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCAGCACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAACAAGTTTGA
451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTTGTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

FIGURE 17B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGTTTAAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGA-----
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCT-----

901 -----CCAGGACGAGTAGTCGGCGGG-----
-----GGTCTGTCTCATCAGCCGCC-----

951 ----GGCTGTATGGATCCTGAGCCCATAGTGCCTATCGTAGGTCGAAATG
----CCGACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTTT
GTCAACACCGGTACGTTTACGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCTAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATAACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

FIGURE 17B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAAGTGTGTGTCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1813.

Sequence name: pAP322

Note: Nucleotides in bold are found within the mutant preprorcin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 17C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP322 (UPA) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q P S L L I R P V V P N P N A D V C M D P E -B chain
PAP322 (UPA) linker:	A chain- C G - - - - P G R V V G G - - - - G C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

FIGURE 18A

Sequence of pAP323 (MMP-9) Linker Region

WT preporicin linker

primer 323-3'
 5' - ATTGCAGGGCAG---GGGGGTAGTAGCGGGGGGGATGTATGGATCCTGAG -3'
 ***** ** ** ** **
 -CTCATGGGTATAGATGGGCACCTCCACCATCGTCACAGTTTCTTGTCTT | ATAAGGCCACAGTGGTACCAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
 -GAGTACCACATATCTACGGGTGGAGGTGGTAGCAGTGTCAAAAGAAACGAA | TATTCGGGTACCATGCTTTAAATTACGACTACAAACATACCTAGGACTCGGG-
 * ***** ** * ** * *
 3' -TACCACATATCTACGCCTCCGCCCTGAGGT---CCCCCAGGCGGTTCT -5'
 primer 323-5'

1) PCR mutagenesis

2) Ligate with pVL1393

pAP323 linker (MMP-9 variant)

GGAGGGGGGACTCCAGGGGGTCCGCAAGGA | ATTGCAGGGCAGGGGGGTAGTAGCGGGGGGA
 CCTCCGCCCTGAGGTCCCCCAGGCGTTCCT | TAACGTCCCGTCCCCCATCATCGCCGCCCT

Note: Nucleotides in bold are found within the preporicin linker region. The '.' symbol within the linker designate deleted nucleotides.

FIGURE 18B (P1)

Sequence of pAP323 insert

10 20 30 40 50
| | | | |
1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA
51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC
101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTAAATATTTGAAATGGTGT
151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTGCGGG
CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC
201 TCGTTTAACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT
251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTAGTTGAACTCTCA
TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAATCAACTTGAGAGT
301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT
351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTTCATCCTGACA
ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT
401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA
451 CGATATACATTGCGCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG
501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC
551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA
601 CTGGCTCGTTCCTTTATAATTTGCATCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTT
651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

FIGURE 18B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGCGGGACT
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCCTCCGCCCTGA

901 CCAGGG---GGTCCGCAAGGAATTGCAGGGCAG---GGGGGTAGTAGCGG
GGTCCC---CCAGGCGTTCCTTAACGTCCCGTC---CCCCCATCATCGCC

951 CGGGGGATGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
GCCCCCTACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGTTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCAAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGA CTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAATAACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

FIGURE 18B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAAACGATGGATGT
ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGAT
AGTTCTTACTACCTTGGTAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1849.

Sequence name: pAP323

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

FIGURE 18C

Amino acid sequence Comparison of Mutant Preproricin Linker Region of PAP323 (MMP-9) to Wild Type

Wild type ricin linker:	A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E -B chain
PAP323 (MMP-9) linker:	A chain- C G G G S S - G G P Q G I A G Q - G G S S G G G C M D P E -B chain

Note: Amino acids in bold are found within the preproricin linker region. The '-' symbol within the linker designate deleted amino acids.

FIGURE 19A

Sequence of pAP324 (MMP-9) Linker Region

WT preporicrin linker

primer 324-3'
 5' - ATTGCAGGGCAG-----GGTAGTAGCGCGGGGATGTATGGATCCTGAG -3'
 ***** ** ** ** **
 -CTCATGGTGTATAGATGCGGCACCTCCACCATCGTCACAGTTTCTTTGCTT|ATAAGGCCAGTGGGTACCAAAATTTTAAATGCTGATGTTGTATGGATCCTGAGCCC-
 -GAGTACCACATATCTACCGGTGGAGGTGGTAGCAGTGTCAAAAGAAACGAA|TATTCGGGTCCACATGGTTTAAAAATTACGACTACAAACATACCCTAGGACTCGGG-
 * ***** ** * ** * *****
 3' -TACCACATATCTACGCCTCCGCCCTGAGGT-----CCAGGGGTTTCCT -5'
 primer 324-5'

1) PCR mutagenesis

2) Ligate with pVL1393

AP324 linker (MMP-9 variant)

GGAGGGGGACTCCAGGTCCGCAAGGA|ATTGCAGGGCAGGGTAGTAGCGGGCGGGGA
 CCTCGCCCTGAGGTCCAGGCTTCCT|TAACGTCCCGTCCCATCATCGCCGCCCT

Note: Nucleotides in bold are found within the preporicrin linker region. The '-' symbol within the linker designate deleted nucleotides.

FIGURE 19B (P1)

Sequence of pAP324 insert

```

      10      20      30      40      50
      |      |      |      |      |
1  GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
   CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51  GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
   CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTATC

101 AGGATAACAACATATTCCCCAACAATACCCAATTATAAACTTTACCACA
   TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
   CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACTGGAGCTGATGTGAGACATGAAATACCAGTGTGCCAA
   AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
   TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
   TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
   ACACCAGCCGATGGCAGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAT
   TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGAAGTCAAGTTTTA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
   GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACCTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
   ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
   TATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
   GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTGCTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
   TAAGGTTATATACTCCCTCTTACGCGTGCTCTTAATCCATGTTGGCCT
```

FIGURE 19B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGCGGGACT
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCGCCCTGA

901 CCA-----GGTCCGCAAGGAATTGCAGGGCAG-----GGTAGTAGCGG
GGT-----CCAGGCGTTCCTTAACGTCCCGTC-----CCATCATCGCC

951 CGGGGGATGTATGGATCCTGAGCCCATAGTGCATATCGTAGGTGAAATG
GCCCCCTACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGAAACGCAATA
CAGATACACAACACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTACAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTGTTACAACCATGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTTCGTTTATCACCTGTTTATACCTATCTCCTGACATCGTCACTTT

FIGURE 19B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCACTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAACATTTAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1843.

Sequence name: pAP324

Note: Nucleotides in bold are found within the mutant preprorcin linker region. The '-' symbol within the linker region designate deleted nucleotides.

[illegible]

Wild type ricin linker: A chain- C A P P S S Q F S L L I R P V V P N F N A D V C M D P E - B chain

PAP324 (WMP-9) linker: A chain- C G G G S S - - G P Q G I A G Q - - G S S G G G C M D P E - B chain

Note: Amino acids in bold are found within the preprocrin linker region. The ‘-’ symbol within the linker designate deleted amino acids.

FIGURE 20A

Sequence of pAP325 (MMP-9) Linker Region

WT preprorincin linker

```

                    primer 325-3'
5' - ATTGCAGGGCAG-----AGTAGCGGGGGGATGTATGGATCCTGAG -3'
      *****
      ** ** ** **
-CTCATGGGTATAGATGCCGACCTCCACCATCGTCACAGTTTCTTTGCTT|ATAAGGCCAGTGGGTACCAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCC-
-GAGTACCAACATATCTACGCGTGGAGGTGGTAGCAGTGTCAAAAGAAACGAA|TATTCGGTCCACCATGGTTTAAATTTACGACTACAAACATACCTAGGACTCGGG-
      * ***** *
3' -TACCACATATCTAGCCCTCCGCCCTGAGGT-----GGCGTTCTCT -5'
                    primer 325-5'

```

1) PCR mutagenesis

2) Ligate with pVL1393

pAP325 linker (MMP-9 variant)

```

GGAGGCGGGACTCCACGCAAGGA|ATTGCGGGGCGAGTAGCGGCGGGGGA
CCTCCGCCCTGAGGTGGCGTTCTCT|TAACGTCCCGTCTCATCGCCGCCCCCT

```

Note: Nucleotides in bold are found within the preprorincin linker region. The '-' symbol within the linker designate deleted nucleotides.

FIGURE 20B (P1)

Sequence of pAP325 insert

10 20 30 40 50

1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
CCGTTGTACCGAAACAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTTCCCAACAATACCCAATTATAAACTTTACCACA
TCCTATTGTTGTATAAGGGGTTTGTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAACTTTATCAGAGCTGTTTCGCGG
CGCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAA
AGCAAATTGTTGACCTCGACTACACTCTGTACTTTATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
TGTCTCAACCAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
ACACCAGCCGATGGCAGGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
TAGTCCTTCTACGTCTTCGTAGTAGTAGAAAAGTGAAGTACAAGTTTGA

451 CGATATACATTTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACCTTGC
GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAAGTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAAC
GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCTGTT

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAAATTAGGTACAACCGGA
TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

FIGURE 20B (P2)

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
CTAGACGTGGTCTAGGATCGCATTAAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
AGTTGACGTTTCTGCATTACCAAGGTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGGAGGCGGGACT
ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCCTCCGCCCTGA

901 CCA-----CCGCAAGGAATTGCAGGGCAG-----AGTAGCGG
GGT-----GGCGTTCCTTAACGTCCCGTC-----TCATCGCC

951 CGGGGGATGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
GCCCCCTACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAACGCAATA
CAGATACACAACCTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
GTCAACACCGGTACGTTTCAAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCAACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
AATGTACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTACAAACATTGTTGGGCTATATGGTCTGTG
TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
GAACGTTGTTTATCACCTGTTTACATACCTATCTCCTGACATCGTCACTTT

FIGURE 20B (P3)

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
ACAATTCTAGGAGAGAACACCCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTAGAT
AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCCAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAATATGGTTACCATTATTTTGATAGACAGATTACT
ACCACTGGGTTTGGTTTATACCAATGGTAATAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAAGTAAAGGACAGCAAGTTATATCGAATTCC
CCTGTAAACATTTAAACATTGACTTTCTGTCTGTTCAATATAGCTTAAGG

1851 TGCAG
ACGTC

Total number of bases is: 1837.

Sequence name: pAP325

Note: Nucleotides in bold are found within the mutant preproricin linker region. The '-' symbol within the linker region designate deleted nucleotides.

Amino acid sequence Comparison of Mutant Preprotrypsin Linker Region of PAP325 (MMP-9) to Wild Type

Note: Amino acids in bold are found within the preprotricin linker region. The ‘-’ symbol within the linker designate deleted amino acids.

FIGURE 21

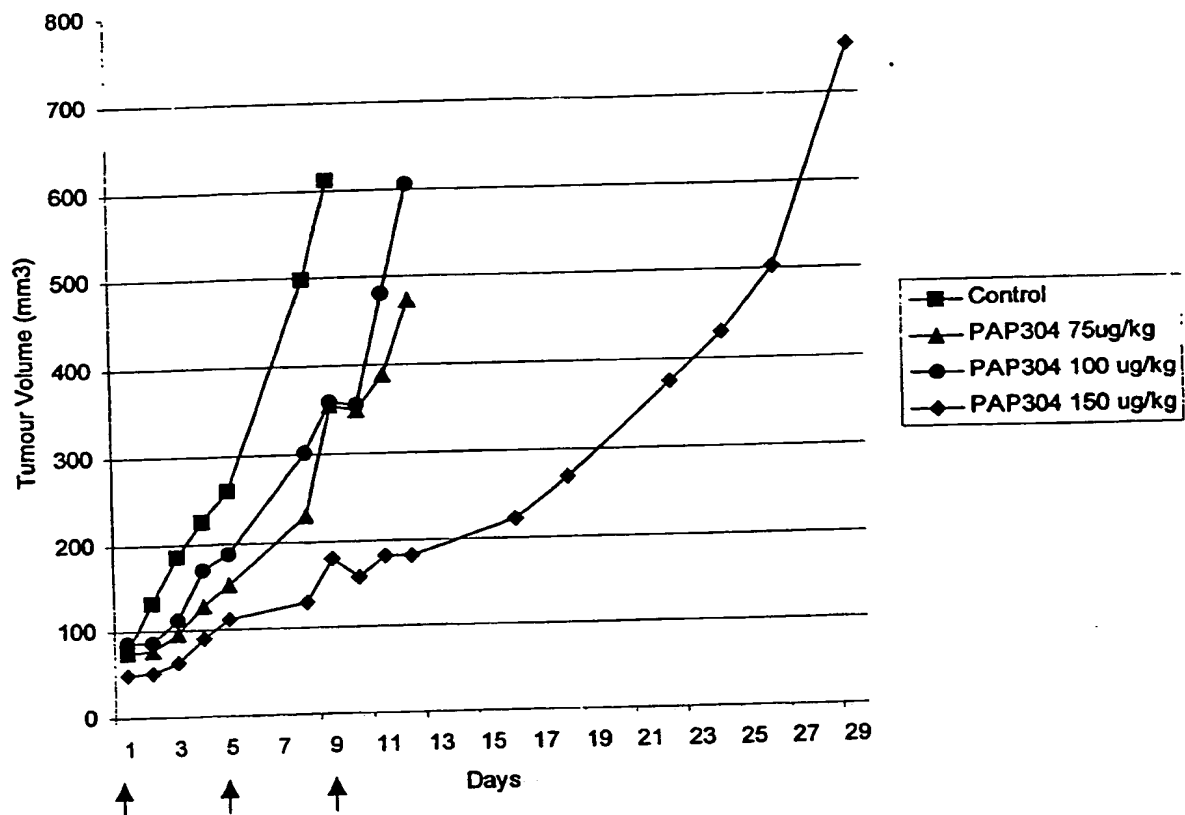
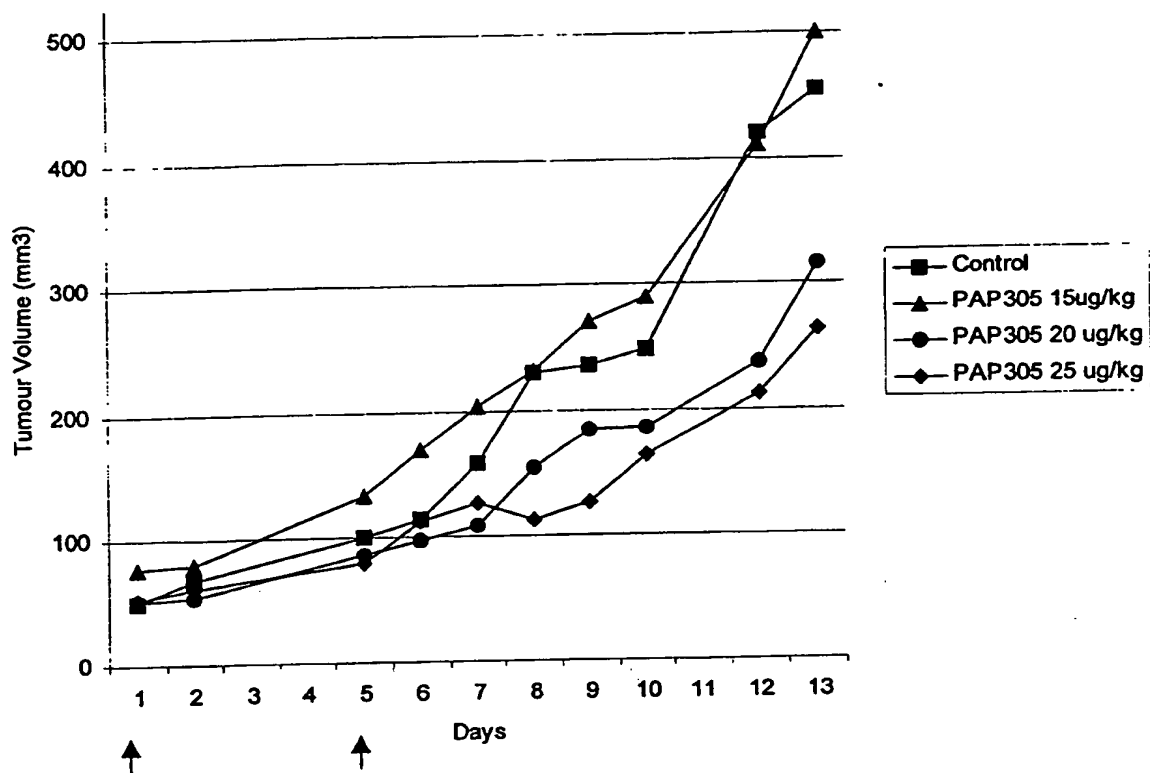


FIGURE 22



THIS PAGE BLANK (USPTO)